

## **JOINT INVENTORS**

# **APPLICATION FOR UNITED STATES LETTERS PATENT**

## **S P E C I F I C A T I O N**

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**TO ALL WHOM IT MAY CONCERN:**

Be it known that we, Kari Alitalo, a citizen of Finland, residing at Nyyrikintie 4A, SF-02100 Espoo, Finland, and Arja Kaipainen, a citizen of Finland, residing at One Emerson Place, Apt. 2M, Boston, Massachusetts 02114, and Reija Valtola, a citizen of Finland, residing at Hilapellontie 2BF1, 00390 Helsinki, Finland, and Lotta Jussila, a citizen of Finland, residing in Helsinki, Finland, have invented a new and useful Flt4 (VEGFR-3) as a Target for Tumor Imaging and Anti-Tumor Therapy, of which the following is a specification.

## **Flt4 (VEGFR-3) AS A TARGET FOR TUMOR IMAGING AND ANTI-TUMOR THERAPY**

This application is a continuing application which claims priority from United States Patent Application Serial No. 08/901,710, filed July 28, 1997; and from  
5 U.S. Patent Application Serial No. 08/340,011, filed November 14, 1994, now U.S. Patent No. 5,776,755; and from United States Patent Application Serial No. 08/257,754, filed July 9, 1994, now abandoned; the latter two of which in turn are continuations-in-part of United States Patent Application Serial No. 07/959,951, filed on October 9, 1992, now abandoned. All of these applications are incorporated herein  
10 by reference, in their entireties.

### **Field of the Invention**

The present invention relates generally to genes for receptors, specifically genes for receptor tyrosine kinases, their insertion into recombinant DNA vectors, and the production of the resulting proteins in host strains of microorganisms  
15 and host eukaryotic cells. More specifically, the present invention is directed to Flt4, a receptor tyrosine kinase; to nucleotide sequences encoding Flt4; to methods for the generation of DNAs encoding Flt4 and their gene products; to nucleic acid probes which specifically recognize (hybridize to) nucleic acids encoding such receptors; to antibodies that specifically recognize such receptors; and to methods of using such  
20 probes and antibodies, e.g., for identifying lymphatic vessels and high endothelial venules (HEV) in animal and human tissues and augmenting or preventing their growth in pathological conditions.

### **BACKGROUND**

The cellular behavior responsible for the development, maintenance and  
25 repair of differentiated cells and tissues is regulated, in large part, by intercellular signals conveyed via growth factors and similar ligands and their receptors. The receptors are located on the cell surface of responding cells and they bind peptides or polypeptides known as growth factors as well as other hormone-like ligands. The

results of this interaction are rapid biochemical changes in the responding cells, as well as a rapid and a long term readjustment of cellular gene expression. Several receptors associated with various cell surfaces can bind specific growth factors.

5 Tyrosine phosphorylation is one of the key modes of signal transduction across the plasma membrane. Several tyrosine kinase genes encode transmembrane receptors for polypeptide growth factors and hormones, such as epidermal growth factor (EGF), insulin, insulin-like growth factor-I (IGF-I), platelet derived growth factors (PDGF-A and -B) and fibroblast growth factors (FGFs) [Heldin et al., *Cell Regulation*, 1: 555-566 (1990); Ullrich et al., *Cell*, 61: 243-54 (1990)]. The receptors  
10 of several hematopoietic growth factors are tyrosine kinases; these include c-fms, which is the colony stimulating factor 1 receptor [Sherr et al., *Cell*, 41: 665-676 (1985)] and c-kit, a primitive hematopoietic growth factor receptor [Huang et al., *Cell*, 63: 225-33 (1990)].

These receptors differ in their specificity and affinity. In general,  
15 receptor tyrosine kinases are glycoproteins, which consist of an extracellular domain capable of binding a specific growth factor(s), a transmembrane domain which is usually an alpha-helical portion of the protein, a juxtamembrane domain (where the receptor may be regulated by, e.g., protein phosphorylation), a tyrosine kinase domain (which is the enzymatic component of the receptor), and a carboxy-terminal tail, which  
20 in many receptors is involved in recognition and binding of the substrates for the tyrosine kinase.

In several receptor tyrosine kinases, the processes of alternative splicing and alternative polyadenylation are capable of producing several distinct polypeptides from the same gene. These may or may not contain the various domains listed above.  
25 As a consequence, some extracellular domains may be expressed as separate proteins secreted by the cells and some forms of the receptors may lack the tyrosine kinase domain and contain only the extracellular domain inserted into the plasma membrane via the transmembrane domain plus a short carboxy-terminal tail.

The physiology of the vascular system, embryonic vasculogenesis and  
30 angiogenesis, blood clotting, wound healing and reproduction, as well as several

diseases, involve the vascular endothelium lining the blood vessels. The development of the vascular tree occurs through angiogenesis, and, according to some theories, the formation of the lymphatic system starts shortly after arterial and venous development by sprouting from veins. See Sabin, F.R., *Am. J. Anat.*, 9:43 (1909); and van der Putte, S.C.J., *Adv. Anat. Embryol. Cell Biol.*, 51:3 (1975).

After the fetal period, endothelial cells proliferate very slowly, except during angiogenesis associated with neovascularization. Growth factors stimulating angiogenesis exert their effects via specific endothelial cell surface receptor tyrosine kinases.

Among ligands for receptor tyrosine kinases, the Platelet Derived Growth Factor (PDGF) has been shown to be angiogenic, albeit weakly, in the chick chorioallantoic membrane. Transforming Growth Factor  $\alpha$  (TGF $\alpha$ ) is an angiogenic factor secreted by several tumor cell types and by macrophages. Hepatocyte Growth Factor (HGF), the ligand of the c-met proto-oncogene-encoded receptor, is also strongly angiogenic, inducing similar responses to those of TGF $\alpha$  in cultured endothelial cells.

Striking new evidence shows that there are endothelial cell specific growth factors and receptors that may be primarily responsible for the stimulation of endothelial cell growth, differentiation, as well as certain of differentiated functions.

The most-widely studied growth factor is Vascular Endothelial Growth Factor (VEGF), a member of the PDGF family. Vascular endothelial growth factor is a dimeric glycoprotein of disulfide-linked 23 kDa subunits, discovered because of its mitogenic activity toward endothelial cells and its ability to induce vessel permeability (hence its alternative name vascular permeability factor). Other reported effects of VEGF include the mobilization of intracellular  $\text{Ca}^{2+}$ , the induction of plasminogen activator and plasminogen activator inhibitor-1 synthesis, stimulation of hexose transport in endothelial cells, and promotion of monocyte migration *in vitro*. Four VEGF isoforms, encoded by distinct mRNA splicing variants, appear to be equally capable of stimulating mitogenesis of endothelial cells. The 121 and 165 amino acid isoforms of VEGF are secreted in a soluble form, whereas the isoforms of 189 and 206

amino acid residues remain associated with the cell surface and have a strong affinity for heparin. Soluble non-heparin-binding and heparin-binding forms have also been described for the related placenta growth factor (PlGF; 131 and 152 amino acids, respectively), which is expressed in placenta, trophoblastic tumors, and cultured human  
5 endothelial cells.

The pattern of VEGF expression suggests its involvement in the development and maintenance of the normal vascular system and in tumor angiogenesis. During murine development, the entire 7.5 day post-coital endoderm expresses VEGF and the ventricular neuroectoderm produces VEGF at the capillary  
10 ingrowth stage. On day two of quail development, the vascularized area of the yolk sac as well as the whole embryo show expression of VEGF. In addition, epithelial cells next to fenestrated endothelia in adult mice show persistent VEGF expression, suggesting a role in the maintenance of this specific endothelial phenotype and function.

Two high affinity receptors for VEGF have been characterized, VEGFR-1/Flt1 (fms-like tyrosine kinase-1) and VEGFR-2/Kdr/Flk-1 (kinase insert domain containing receptor/fetal liver kinase-1). These receptors are classified in the PDGF-receptor family. However, the VEGF receptors have seven  
15 immunoglobulin-like loops in their extracellular domains (as opposed to five in other members of the PDGF family) and a longer kinase insert. The expression of VEGF  
20 receptors occurs mainly in vascular endothelial cells, although some may also be present on monocytes and on melanoma cell lines. Only endothelial cells have been reported to proliferate in response to VEGF, and endothelial cells from different sources show different responses. Thus, the signals mediated through VEGFR-1 and  
25 VEGFR-2 appear to be cell type specific.

VEGFR-1 and VEGFR-2 bind VEGF<sub>165</sub> with high affinity ( $K_d$  about 20 pM and 200 pM, respectively). Flk-1 receptor has also been shown to undergo autophosphorylation in response to VEGF, but phosphorylation of Flt1 was barely detectable. VEGFR-2 mediated signals cause striking changes in the morphology,  
30 actin reorganization and membrane ruffling of porcine aortic endothelial cells

overexpressing this receptor. In these cells, VEGFR-2 also mediated ligand-induced chemotaxis and mitogenicity; whereas VEGFR-1 transfected cells lacked mitogenic responses to VEGF. In contrast, VEGF had a strong growth stimulatory effect on rat sinusoidal endothelial cells expressing VEGFR-1. Phosphoproteins co-precipitating  
5 with VEGFR-1 and VEGFR-2 are distinct, suggesting that different signalling molecules interact with receptor specific intracellular sequences.

In *in situ* hybridization studies, mouse VEGFR-2 mRNA expression was found in yolk sac and intraembryonic mesoderm (estimated 7.5 day post-coitum (p.c.) embryos, from which the endothelium is derived, and later in presumptive  
10 angioblasts, endocardium and large and small vessel endothelium (12.5 days p.c.). Abundant VEGFR-2 mRNA in proliferating endothelial cells of vascular sprouts and branching vessels of embryonic and early postnatal brain and decreased expression in adult brain suggested that VEGFR-2 is a major regulator of vasculogenesis and angiogenesis. VEGFR-1 expression was similarly associated with early vascular  
15 development in mouse embryos and with neovascularization in healing skin wounds. However, high levels of VEGFR-1 expression were detected in adult organs, suggesting that VEGFR-1 has a function in quiescent endothelium of mature vessels not related to cell growth. The avian homologue of VEGFR-2 was observed in the mesoderm from the onset of gastrulation, whereas the VEGFR-1 homologue was first  
20 found in cells co-expressing endothelial markers. In *in vitro* quail epiblast cultures, FGF-2, which is required for vasculogenic differentiation of these cells, upregulated VEGFR-2 expression. The expression of both VEGF receptors was found to become more restricted later in development. In human fetal tissues VEGFR-1 and VEGFR-2 showed overlapping, but slightly different, expression patterns. These data suggest  
25 that VEGF and its receptors act in a paracrine manner to regulate the differentiation of endothelial cells and neovascularization of tissues.

VEGF recently has been shown to be a hypoxia-induced stimulator of endothelial cell growth and angiogenesis, and inhibition of VEGF activity using specific monoclonal antibodies has been shown to reduce the growth of experimental  
30 tumors and their blood vessel density. [Ferrara *et al.*, *Endocrine Reviews*, 18: 4-25

(1997); Shibuya *et al.*, *Adv. Cancer Res.*, 67: 281-316 (1995); Kim *et al.*, *Nature*, 362: 841-844 (1993).]

Growth of solid tumors beyond a few cubic millimeters in size is dependent on vascular supply, making angiogenesis an attractive target for anti-cancer therapy. Encouraging results have been reported with endogenous angiogenic inhibitors or "statins" which include angiostatin, a fragment of plasminogen, and endostatin, a fragment of collagen 18. [O'Reilly *et al.*, *Cell*, 79: 315-328 (1994); O'Reilly *et al.*, *Cell*, 88: 277-85 (1997).]. Both factors are normally produced by primary tumors and keep metastasis dormant. Systemic administration of either statin has been shown to also induce and sustain dormancy of primary tumors in animal models. The receptors and signalling by statins, as well as the proteases which activate them, remain to be identified. A need exists for additional therapeutic molecules for controlling angiogenesis in the treatment of cancer and other pathological disease states.

Primary breast cancers have been shown to express several angiogenic polypeptides, of which VEGF was the most abundant. [See, e.g., Relf *et al.*, *Cancer Res.*, 57: 963-969 (1997).] Tumor cells contained high levels of VEGF mRNA in both invasive and non-invasive, ductal (*in situ*) breast carcinomas. [Brown *et al.*, *Hum. Pathol.*, 26: 86-91 (1995).] The endothelial cells adjacent to the *in situ* carcinomas expressed VEGFR-1 and VEGFR-2 mRNA. VEGF and its receptors may contribute to the angiogenic progression of malignant breast tumors, because in several independent studies, correlations have been found between tumor vascular density and the prognosis of the disease. [Weidner *et al.*, *J. Natl. Cancer Inst.*, 84: 1875-1887 (1992).] A need exists for additional markers for breast cancer and breast cancer-related angiogenesis, to improve diagnosis and screening and to serve as a target for therapeutic intervention.

A major function of the lymphatic system is to provide fluid return from tissues and to transport many extravascular substances back to the blood. In addition, during the process of maturation, lymphocytes leave the blood, migrate through lymphoid organs and other tissues, and enter the lymphatic vessels, and return to the

blood through the thoracic duct. Specialized venules, high endothelial venules (HEVs), bind lymphocytes again and cause their extravasation into tissues. The lymphatic vessels, and especially the lymph nodes, thus play an important role in immunology and in the development of metastasis of different tumors.

5                Since the beginning of the 20th century, three different theories concerning the embryonic origin of the lymphatic system have been presented. However, lymphatic vessels have been difficult to identify, due to the absence of known specific markers available for them.

10              Lymphatic vessels are most commonly studied with the aid of lymphography. In lymphography, X-ray contrast medium is injected directly into a lymphatic vessel. That contrast medium is distributed along the efferent drainage vessels of the lymphatic system. The contrast medium is collected in lymph nodes, where it stays for up to half a year, during which time X-ray analyses allow the follow-up of lymph node size and architecture. This diagnostic is especially important in  
15              cancer patients with metastases in the lymph nodes and in lymphatic malignancies, such as lymphoma. However, improved materials and methods for imaging lymphatic tissues are needed in the art.

### **SUMMARY OF THE INVENTION**

20              The present invention addresses a gene for a novel receptor tyrosine kinase located on chromosome 5, identified as an unknown tyrosine kinase-homologous PCR-cDNA fragment from human leukemia cells [Aprelikova et al., *Cancer Res.*, 52: 746-748 (1992)]. This gene and its encoded protein are called *Flt4*. This abbreviation comes from the words *fms*-like tyrosine kinase 4.

25              Flt4 is a receptor tyrosine kinase closely related in structure to the products of the VEGFR-1 and VEGFR-2 genes. By virtue of this similarity and subsequently-discovered similarities between ligands for these three receptors, the Flt4 receptor has additionally been named VEGFR-3. The names Flt4 and VEGFR-3 are used interchangeably herein. Despite the similarity between these three receptors, the mature form of Flt4 differs from the VEGFRs in that it is proteolytically cleaved in the



extracellular domain into two disulfide-linked polypeptides of 125/120 kD and 75 kD. The *Flt4* gene encodes 4.5 and 5.8 kb mRNAs which exhibit alternative 3' exons and encode polypeptides of 190 kD and 195 kD, respectively.

Further evidence of a distinction is that VEGF does not show specific  
5 binding to Flt4 and doesn't induce its autophosphorylation.

A comparison of *Flt4*, Flt1, and KDR/Flk-1 receptor mRNA signals showed overlapping, but distinct expression patterns in the tissues studied. Kaipainen, *et al.*, *J. Exp. Med.*, 178:2077 (1993). *Flt4* gene expression appears to be more restricted than the expression of VEGFR-1 or VEGFR-2. The expression of *Flt4* first  
10 becomes detectable by *in situ* hybridization in the angioblasts of head mesenchyme, the cardinal vein and extraembryonically in the allantois of 8.5 day post-coital mouse embryos. In 12.5 day post-coital embryos the *Flt4* signal is observed on developing venous and presumptive lymphatic endothelia, but arterial endothelia appear to be negative. During later stages of development, *Flt4* mRNA becomes restricted to  
15 developing lymphatic vessels. Only the lymphatic endothelia and some high endothelial venules express *Flt4* mRNA in adult human tissues and increased expression occurs in lymphatic sinuses in metastatic lymph nodes and in lymphangioma. The results support the theory of the venous origin of lymphatic vessels.

The protein product of the *Flt4* receptor tyrosine kinase cDNA, cloned  
20 from a human erythroleukemia cell line, is N-glycosylated and contains seven immunoglobulin-like loops in its extracellular domain. The cytoplasmic tyrosine kinase domain of Flt4 is about 80 % identical at the amino acid level with the corresponding domains of Flt1 and KDR and about 60 % identical with the receptors for platelet-derived growth factor, colony stimulating factor-1, stem cell factor, and the Flt3  
25 receptor. See Pajusola *et al.*, *Cancer Res.*, 52:5738 (1992).

The present invention provides isolated polynucleotides (*e.g.*, DNA or RNA segments of defined structure) encoding an Flt4 receptor tyrosine kinase useful in the production of Flt4 protein and peptide fragments thereof and in recovery of related genes from other sources.

The present invention provides a recombinant DNA vector containing a heterologous segment encoding the Flt4 receptor tyrosine kinase or a related protein that is capable of being inserted into a microorganism or eukaryotic cell and that is capable of expressing the encoded protein.

5               The present invention provides eukaryotic cells capable of producing useful quantities of the Flt4 receptor tyrosine kinase and proteins of similar function from many species.

              The present invention provides peptides that may be produced synthetically in a laboratory or by microorganisms, which peptides mimic the activity of  
10           the natural Flt4 receptor tyrosine kinase protein. In another embodiment, the invention is directed to peptides which inhibit the activity of Flt4 receptor tyrosine kinase protein.

              Particularly preferred are peptides selected from the group consisting of: (a) a *Flt4*-short form, the nucleotide and deduced amino acid sequence of which  
15           appear in SEQ. ID NOs. 1 and 2; and (b) a second form with different nucleotide and corresponding amino acid residues at its carboxyl terminal, i.e., an *Flt4*-long form, the nucleotide and deduced amino acid sequence of which appear in SEQ. ID NOs. 3 and 4. The Flt4 long form has a length of 1363 amino acid residues.

              DNA and RNA molecules, recombinant DNA vectors, and modified  
20           microorganisms or eukaryotic cells comprising a nucleotide sequence that encodes any of the proteins or peptides indicated above are also part of the present invention. In particular, sequences comprising all or part of the following two DNA sequences, a complementary DNA or RNA sequence, or a corresponding RNA sequence are especially preferred: (a) a DNA sequence such as SEQ ID NO: 1, encoding *Flt4*-short  
25           form [SEQ ID NO: 2], and (b) a DNA sequence such as SEQ ID NO: 3, encoding a Flt4 wherein nucleotides 3913-4416 of SEQ ID NO: 1 are changed, encoding *Flt4*-long form [SEQ ID NO: 4].

              DNA and RNA molecules containing segments of the larger sequence are also provided for use in carrying out preferred aspects of the invention relating to

the production of such peptides by the techniques of genetic engineering and the production of oligonucleotide probes.

Because the DNA sequence encoding the Flt4 protein is identified herein, DNA encoding the Flt4 protein may be produced by, e.g., polymerase chain  
5 reaction or by synthetic chemistry using commercially available equipment, after which the gene may be inserted into any of the many available DNA vectors using known techniques of recombinant DNA technology. Furthermore, automated equipment is also available that makes direct synthesis of any of the peptides disclosed herein readily available.

10 The present invention also is directed to Flt4 peptides and other constructs and to the use of Flt4 as a specific marker for lymphatic endothelial cells.

In a specific embodiment, the invention is directed to nucleic acid probes and antibodies recognizing Flt4, especially to monoclonal antibodies, and compositions containing such antibodies.

15 Also in a specific embodiment, the invention is directed to a method for monitoring lymphatic vessels in tissue samples and in organisms. Further, is it an object of the present invention to provide clinical detection methods describing the state of lymphatic tissue, and especially lymphatic vessels (inflammation, infection, traumas, growth, etc.), and to provide methods for detecting lymphatic vessels, and  
20 thus lymphatic vascularization, in an organism.

It is a further object of the present invention to provide monoclonal antibodies which specifically recognize the Flt4 receptor protein or various epitopes thereof. It is an object of the invention to use these monoclonal antibodies for diagnostic purposes for detecting and measuring the amount of Flt4 receptors in  
25 tissues, especially in lymphatic tissues.

Another aspect of the present invention relates to a method of determining the presence of Flt4-receptors in a cell sample, comprising the steps of: (a) exposing a cell sample to an antibody, especially a monoclonal antibody, of the present invention; and (b) detecting the binding of said monoclonal antibody to Flt4 receptors.

The invention is further directed to a method of modulating (e.g., antagonizing or augmenting) the function of Flt4 in lymphatic vascularization and in inflammatory, infectious and immunological conditions. For example, in one embodiment, such a method comprises inhibiting the Flt4-mediated lymphatic  
5 vascularization by providing amounts of a Flt4-binding compound sufficient to block the Flt4 endothelial cell sites participating in such reaction, especially where Flt4 function is associated with a disease such as metastatic cancers, lymphomas, inflammation (chronic or acute), infections and immunological diseases.

The invention is further directed to a specific Flt4-stimulating ligand  
10 and monoclonal antibodies and their use for stimulating lymphatic endothelia and fragments and peptides as well as antibodies derived from research on the ligand to inhibit Flt4 function when desirable, such as in various disease states involving Flt4 function.

The invention provides a cell line source for the ligand of the Flt4  
15 receptor tyrosine kinase. Using the conditioned medium from these cells, the Flt4 ligand may be purified and cloned by using methods standard in the art. Using this conditioned medium or the purified ligand, an assay system for Flt4 ligand and dimerization inhibitors as well as inhibitors of Flt4 signal transduction are obtained, which allow for identification and preparation of such inhibitors.

20 In a preferred embodiment of the invention, conditioned medium from the PC-3 cell line comprises a protein or a fragment thereof, which is capable of stimulating the Flt4 receptor and regulating the growth and differentiation as well as the differentiated functions of certain endothelial cells. The Flt4 ligand or its peptides or derivatives are useful in the regulation of endothelial cell growth, differentiation and  
25 their differentiated functions and in the generation of agonists and antagonists for the ligand. Particularly, the Flt4 ligand is useful in regulating lymphatic endothelia. However, the Flt4 ligand, when purified, or produced from a recombinant source, may also stimulate the related KDR/Flk-1 receptor.

The identification of Flt4-stimulating ligand makes it directly possible to  
30 assay for inhibitors of this ligand or inhibitors of Flt4 function. Such inhibitors are

simply added to the conditioned media containing the Flt4 ligand and if they inhibit autophosphorylation, they act as Flt4 signalling inhibitors. For example, recombinant or synthetic peptides (including but not limited to fragments of the Flt4 extracellular domain) may be assayed for inhibition of Flt4-ligand interaction or Flt4 dimerization.

5 Such putative inhibitors of Flt4 and, in addition, antibodies against the Flt4 ligand, peptides or other compounds blocking Flt4 receptor-ligand interaction, as well as antisense oligonucleotides complementary to the sequence of mRNA encoding the Flt4 ligand are useful in the regulation of endothelial cells and in the treatment of diseases associated with endothelial cell function.

10 A detailed characterization of the Flt4 ligand, designated VEGF-C, is provided in PCT Patent Application No. PCT/US98/01973, filed 2 February 1998, and published as International Publication No. WO 98/33917; in PCT Patent Application PCT/FI96/00427, filed August 1, 1996, and published as International Publication WO 97/05250; and in the U.S. Patent Application priority documents relied upon therein  
15 for priority, all of which are incorporated herein by reference. The deduced amino acid sequence for prepro-VEGF-C is set forth herein in SEQ ID NO: 21.

A detailed description of a second Flt4 ligand, designated VEGF-D, is provided in Achen, *et al.*, *Proc. Nat'l Acad. Sci. U.S.A.*, 95(2): 548-553 (1998), and in Genbank Accession No. AJ000185, both of which are incorporated herein by  
20 reference. The deduced amino acid sequence for prepro-VEGF-D is set forth herein in SEQ ID NO: 22.

The invention also is directed to a method of treating a mammalian organism suffering from a disease characterized by expression of Flt4 tyrosine kinase (Flt4) in cells, comprising the step of administering to the mammalian organism a  
25 composition, the composition comprising a compound effective to inhibit the binding of an Flt4 ligand protein to Flt4 expressed in cells of the organism, thereby inhibiting Flt4 function. The disease may be diseases already mentioned above, such as diseases characterized by undesirable lymphatic vascularization. Additionally, it has been discovered that Flt4 expression also occurs in blood vessel vasculature associated with  
30 at least some breast cancers, and possibly other cancers (*i.e.*, at a level greatly

exceeding the barely detectable or undetectable levels of expression in blood vessel vasculature of corresponding normal (healthy) tissue). Thus, in a preferred embodiment, the cells comprise endothelial cells (lymphatic or vascular). In another embodiment, the cells comprise neoplastic cells such as certain lymphomas that express  
5 Flt4. Treatment of humans is specifically contemplated.

By "compound effective to inhibit the binding of an Flt4 ligand protein to Flt4 expressed in cells of the organism" is meant any compound that inhibits the binding of the Flt4 ligand described herein as vascular endothelial growth factor C, as isolatable from PC-3 conditioned medium. It is contemplated that such compounds  
10 also will be effective for inhibiting the binding of vascular endothelial growth factor D to Flt4. Exemplary compounds include the following polypeptides: (a) a polypeptide comprising an antigen-binding fragment of an anti-Flt4 antibody; (b) a polypeptide comprising a soluble Flt4 fragment (*e.g.*, an extracellular domain fragment), wherein the fragment and the polypeptide are capable of binding to an Flt4 ligand; (c) a  
15 polypeptide comprising a fragment or analog of a vertebrate vascular endothelial growth factor C (VEGF-C) polypeptide, wherein the polypeptide and the fragment or analog bind, but fail to activate, the Flt4 expressed on native host cells; and (d) a polypeptide comprising a fragment or analog of a vertebrate vascular endothelial growth factor-D (VEGF-D) polypeptide, wherein the polypeptide and the fragment or  
20 analog bind, but fail to activate, the Flt4 expressed on native host cells. Small molecule inhibitors identifiable by standard *in vitro* screening assays, *e.g.*, using VEGF-C and recombinantly-expressed Flt4 also are contemplated. Polypeptides comprising an antigen-binding fragment of an anti-Flt4 antibody are highly preferred. Such polypeptides include, *e.g.*, polyclonal and monoclonal antibodies that specifically  
25 bind Flt4; fragments of such antibodies; chimaeric and humanized antibodies; bispecific antibodies that specifically bind to Flt4 and also specifically bind to another antigen, and the like.

In a preferred variation, the compound further comprises a detectable label as described elsewhere herein, or a cytotoxic agent. Exemplary cytotoxic agents  
30 include plant toxins (*e.g.*, ricin, saporin), bacterial or fungal toxins, radioisotopes (*e.g.*,

211-Astatine, 212-Bismuth, 90-Yttrium, 131-Iodine, 99m-Technitium, and others described herein), anti-metabolite drugs (*e.g.*, methotrexate, 5-fluorodeoxyuridine), alkylating agents (*e.g.*, chlorambucil), anti-mitotic agents (*e.g.*, vinca alkaloids), and DNA intercalating agents (*e.g.*, adriamycin).

5                Likewise, to improve administration, the composition preferably further comprises a pharmaceutically acceptable diluent, adjuvant, or carrier medium.

              As explained in detail herein, Flt4 expression, while largely restricted to the lymphatic endothelia of healthy adults, has been identified in the blood vasculature surrounding at least certain tumors. Thus, the invention further includes a method of  
10        treating a mammalian organism suffering from a neoplastic disease characterized by expression of Flt4 tyrosine kinase (Flt4) in vascular endothelial cells, comprising the steps of: administering to a mammalian organism in need of such treatment a composition, the composition comprising a compound effective to inhibit the binding  
15        of an Flt4 ligand protein to Flt4 expressed in vascular endothelial cells of the organism, thereby inhibiting Flt4-mediated proliferation of the vascular endothelial cells.

              Treatment of neoplastic diseases selected from carcinomas (*e.g.*, breast carcinomas), squamous cell carcinomas, lymphomas, melanomas, and sarcomas, are specifically contemplated. However, it will be readily apparent that the screening techniques described herein in detail will identify other tumors characterized by Flt4 expression in  
20        vascular endothelial cells, which tumors are candidates susceptible to the anti-Flt4 treatment regimens described herein. Treatment of breast carcinomas characterized by expression of Flt4 in vascular endothelial cells is specifically contemplated. By neoplastic disease characterized by expression of Flt4 tyrosine kinase in vascular endothelial cells is meant a disease wherein Flt4 is identifiable in blood vasculature at a  
25        much higher level than the undetectable or barely detectable levels normally observed in the blood vascular of healthy tissue, as exemplified herein.

              Therapeutically effective amounts of compounds are empirically determined using art-recognized dose-escalation and dose-response assays. By therapeutically effective for treatment of tumors is meant an amount effective to reduce  
30        tumor growth, or an amount effective to stop tumor growth, or an amount effective to

shrink or eliminate tumors altogether, without unacceptable levels of side effects for patients undergoing cancer therapy. Where the compound comprises an antibody or other polypeptide, doses on the order of 0.1 to 100 mg antibody per kilogram body weight, and more preferably 1 to 10 mg/kg, are specifically contemplated. For  
5 humanized antibodies, which typically exhibit a long circulating half-life, dosing at intervals ranging from daily to every other month, and more preferably every week, or every other week, or every third week, are specifically contemplated. Monitoring the progression of the therapy, patient side effects, and circulating antibody levels will provide additional guidance for an optimal dosing regimen. Data from published and  
10 ongoing clinical trials for other antibody-based cancer therapeutics (e.g., anti-HER2, anti-EGF receptor) also provide useful dosing regimen guidance.

For therapeutic methods described herein, preferred compounds include polypeptides comprising an antigen-binding fragment of an anti-Flt4 antibody, and polypeptides comprising a soluble Flt4 extracellular domain fragment. Human and  
15 humanized anti-Flt4 antibodies are highly preferred.

An expected advantage of the therapeutic methods of the invention lies in the fact that Flt4 is normally not expressed at any significant level in the blood vasculature of healthy tissues. In a highly preferred embodiment, the therapeutic compound comprises a bispecific antibody, or fragment thereof, wherein the antibody  
20 or fragment specifically binds Flt4 and specifically binds a blood vascular endothelial marker antigen. By "blood vascular endothelial marker antigen" is meant any cell surface antigen that is expressed on proliferating vascular endothelial cells, and, preferably, that is not expressed on lymphatic endothelial cells. Exemplary blood vascular endothelial markers include PAL-E [deWaal, *et al.*, *Am. J. Pathol.*, 150:1951-  
25 1957 (1994)], VEGFR-1 and VEGFR-2 [Ferrara *et al.*, *Endocrine Reviews*, 18:4-25 (1997)], Tie [Partanen *et al.*, *Mol. Cell. Biol.*, 12: 1698-1707 (1992)], endoglin [U.S. Patent No. 5,776,427, incorporated herein by reference in its entirety], and von Willebrandt Factor. Such bispecific antibodies are expected to preferentially locate to the tumor-associated vasculature that expresses both Flt4 and the blood vascular  
30 endothelial marker. In a highly preferred embodiment, the compound further



comprises an anti-neoplastic or cytotoxic agent conjugated to the bispecific antibody, for the purposes of killing the tumor cells and/or killing the vasculature supply to the tumor cells. Exemplary agents include those described above, and also therapeutic proteins, such as statins, cytokines, chemokines, and the like, to stimulate an immune response to the tumor in the host.

In an alternative embodiment, the compound comprises an antibody (or bispecific antibody) that recognizes an epitope (or epitopes) comprised of an Flt4/Flt4 ligand complex (e.g., a complex comprised of Flt4 bound to VEGF-C or VEGF-D).

It is further contemplated that the therapeutic compound will be conjugated or co-administered with broad spectrum agents that have potential to inhibit angiogenic factors. Such agents include, e.g., heparin binding drugs (such as pentosan and suramin analogs) that may inhibit angiogenic factors that bind heparin; and chemical agents that block endothelial cell growth and migration, such as fumagillin analogs.

Conjugation of the anti-Flt4 compound to a prodrug that would be targeted to tumor vessels by the anti-Flt4 compound and then activated (e.g., by irradiation) locally at sites of tumor growth also is contemplated. Use of such prodrug strategy has the expected advantage of minimizing side effects of the drug upon healthy lymphatic vessels that express Flt4.

Similarly, the invention includes a method of treating a mammalian organism suffering from a neoplastic disease characterized by expression of Flt4 tyrosine kinase (Flt4) in vascular endothelial cells, comprising the steps of: identifying a mammalian organism suffering from a neoplastic disease state characterized by expression of Flt4 in vascular endothelial cells, and administering to the mammalian organism in need of such treatment a composition, the composition comprising a compound effective to inhibit the binding of an Flt4 ligand protein to Flt4 expressed in vascular endothelial cells of the organism, thereby inhibiting Flt4-mediated proliferation of the vascular endothelial cells.

The invention also provides a method for screening a biological sample for the presence of Flt4 receptor tyrosine kinase protein (Flt4), comprising the steps of:

(a) contacting a biological sample suspected of containing Flt4 with a composition comprising an Flt4 binding compound, under conditions wherein the compound will bind to Flt4 in the biological sample; (b) washing the biological sample under conditions that will remove Flt4 binding compound that is not bound to Flt4 in the sample; and (c) screening the sample for the presence of Flt4 by detecting Flt4 binding compound bound to Flt4 receptor tyrosine kinase in the sample after the washing step. Preferably, the compound comprises a polypeptide selected from the group consisting of: (a) a polypeptide comprising an antigen-binding fragment of an anti-Flt4 antibody; and (b) a polypeptide comprising an Flt4 ligand or Flt4 binding fragment thereof.

Antibodies that specifically bind Flt4, and that further comprise a detectable label, are highly preferred.

The invention also is directed to a method for imaging vertebrate tissue suspected of containing cells that express Flt4 receptor tyrosine kinase protein (Flt4), comprising the steps of: (a) contacting vertebrate tissue with a composition comprising an Flt4 binding compound; and (b) imaging the tissue by detecting the Flt4 binding compound bound to the tissue. Preferably, the tissue is human tissue, and the method further comprises the step of washing the tissue, after the contacting step and before the imaging step, under conditions that remove from the tissue Flt4 compound that is not bound to Flt4 in the tissue.

The invention is further directed to a method of screening for a neoplastic disease state, comprising the steps of: (a) contacting tissue from a mammalian organism suspected of having a neoplastic disease state with a composition comprising an antibody or antibody fragment that specifically binds Flt4 receptor tyrosine kinase; (b) detecting the antibody or antibody fragment bound to cells in the mammalian organism; and (c) screening for a neoplastic disease from the quantity or distribution of the antibody bound to cells in the mammalian organism. As described herein, Flt4 (which usually is undetectable or barely detectable in the blood vasculature) is strongly stained in the blood vasculature of at least some tumors. Thus, in one embodiment, in the screening step, the detection of the antibody or antibody fragment bound to blood vessel endothelial cells is correlated with the presence of a

neoplastic disease. In this method, it will be understood that "detection" means detection at a level significantly higher than the barely detectable or undetectable levels that would occur in corresponding normal (healthy) tissue, as described herein. Such differential expression can be confirmed by comparison to a control performed with tissue from a healthy organism. Screening mammary tissue for neoplasms is specifically contemplated.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

FIG. 1A is a schematic depiction of the structure of *Flt4* cDNA clones;

FIG. 1B is a photographic reproduction of a Northern hybridization gel;

FIGS. 2A-F present a schematic depiction of structural features of Flt4 and a comparison with the Flt1 tyrosine kinase sequence;

FIG. 3A is a schematic depiction of the 3' ends of the cDNA inserts of clones J.1.1 and I.1.1;

FIG. 3B is a photographic reproduction of autoradiograms of hybridizations with anti-sense RNA probe and the long and short forms of *Flt4* RNA;

FIG. 3C is a photographic reproduction of autoradiograms of hybridizations with anti-sense RNA probe and the long and short forms of *Flt4* RNA;

FIG. 4 is a photographic reproduction of a gel illustrating a hybridization analysis of *Flt4* sequences in DNA samples from different species;

FIGS. 5A-5H depict immunohistochemical characterization of VEGFR-3-expressing vessels in intraductal carcinoma. In adjacent sections (FIGS. 5A, B), VEGFR-3 and PAL-E decorate a similar pattern of "necklace" vessels (arrowheads) around the duct filled with carcinoma cells. Another set of adjacent sections was compared with staining for VEGFR-3 (FIG. 5C), laminin (FIG. 5D), collagen XVIII (FIG. 5E) and SMA (FIG. 5F). Double staining for PAL-E and VEGFR-3 (FIG. 5G) and comparison with adjacent section stained for VEGFR-3 only (FIG. 5H). The vessels adjacent to the affected ducts are double-positive (arrowheads), whereas a VEGFR-3 positive vessel is present a short distance away from the affected duct in the interductal stroma (arrows). Note that basal lamina is positive for PAL-E in the

double staining procedure. Magnifications: FIGS. 5A,B 400 x. FIGS. 5C, D, E, F 320 x. FIGS. 5E,F 480 x.

### DETAILED DESCRIPTION

The cloning, sequencing and expression of a novel receptor tyrosine  
5 kinase, termed *Flt4*, is described below. The *Flt4* gene maps to chromosomal region  
5q35 where many growth factors and growth factor receptors are located. The  
extracellular domain of Flt4 consists of seven immunoglobulin-like loops including  
twelve potential glycosylation sites. On the basis of structural similarities, *Flt4* and the  
previously known *Flt1* and *KDR/FLK1* receptors may constitute a subfamily of class  
10 III tyrosine kinases. The *Flt4* gene is expressed as 5.8 kb and 4.5 kb mRNAs which  
were found to differ in their 3' sequences and to be differentially expressed in HEL and  
DAMI leukemia cells.

A Wilm's tumor cell line, a retinoblastoma cell line, and a  
nondifferentiated teratocarcinoma cell line expressed *Flt4*; whereas differentiated  
15 teratocarcinoma cells were negative. Most fetal tissues also expressed the *Flt4*  
mRNA, with spleen, brain intermediate zone and lung showing the highest levels. In  
human adult tissues the highest expression level was found in placenta, lung, kidney,  
heart and liver in decreasing order of expression. In *in situ* hybridization, the *Flt4*  
autoradiographic grains decorated endothelial cells of fetal lung.  
20 Immunohistochemical staining of Flt4 in fetal tissues confirmed staining of the  
endothelial cells. The expression pattern of *Flt4* in comparison to Flt1 and KDR  
differs greatly in tissues of 18-week-old human fetuses. See Kaipainen *et al.*, *J. Exp.*  
*Med.*, 178:2077 (1993).

Expression vectors containing the *Flt4* cDNA have been produced and  
25 expressed in COS and NIH3T3 cells as described in Examples 4 and 11.

The *Flt4* DNAs and polypeptides of the invention may be useful in the  
purification of the Flt4 ligand, and in the regulation of growth and differentiation of  
endothelial cells in various organs. They may also prove valuable in the  
diagnosis/treatment of certain diseases.

In the description that follows, a number of terms used in recombinant DNA (rDNA) technology are extensively utilized. In order to provide a clear and consistent understanding of the specification and claims, including the scope to be given to such terms, the following definitions are provided.

5           Gene. A DNA sequence containing a template for a RNA polymerase. The RNA transcribed from a gene may or may not code for a protein. RNA that codes for a protein is termed messenger RNA (mRNA) and, in eukaryotes, is transcribed by RNA polymerase II. However, it is also known to construct a gene containing a RNA polymerase II template wherein a RNA sequence is transcribed which has a sequence  
10       complementary to that of a specific mRNA but is not normally translated. Such a gene construct is herein termed an "antisense RNA gene" and such a RNA transcript is termed an "antisense RNA." Antisense RNAs are not normally translatable due to the presence of translational stop codons in the antisense RNA sequence.

          A "complementary DNA" or "cDNA" gene includes recombinant genes  
15       synthesized by reverse transcription of mRNA lacking intervening sequences (introns).

Cloning vehicle. A plasmid or phage DNA or other DNA sequence which is able to replicate autonomously in a host cell, and which is characterized by one or a small number of endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without loss of an essential biological function of  
20       the vehicle, and into which DNA may be spliced in order to bring about its replication and cloning. The cloning vehicle may further contain a marker suitable for use in the identification of cells transformed with the cloning vehicle. Markers, for example, are tetracycline resistance or ampicillin resistance. The word "vector" is sometimes used for "cloning vehicle."

25           Expression vector. A vehicle or vector similar to a cloning vehicle and which is capable of expressing a gene which has been cloned into it, after transformation into a host. The cloned gene is usually placed under the control of (i.e., operably linked to) certain control sequences such as promoter sequences. Expression control sequences vary depending on whether the vector is designed to express the  
30       operably linked gene in a prokaryotic or eukaryotic host and may additionally contain

transcriptional elements such as enhancer elements, termination sequences, tissue-specificity elements, and/or translational initiation and termination sites.

The present invention pertains to both expression of recombinant Flt4 proteins (short and long forms), and to the functional derivatives of these proteins.

5            Functional Derivative. A "functional derivative" of Flt4 proteins is a protein which possesses a biological activity (either functional or structural) that is substantially similar to a biological activity of non-recombinant Flt4 proteins. A functional derivative of the Flt4 protein may or may not contain post-translational modifications such as covalently linked carbohydrate, depending on the necessity of  
10 such modifications for the performance of a specific function. The term "functional derivative" is intended to include the "fragments," "variants," "analogues," and "chemical derivatives" of a molecule.

As used herein, a molecule is said to be a "chemical derivative" of another molecule when it contains additional chemical moieties not normally a part of  
15 the molecule. Such moieties may improve the molecule's solubility, absorption, biological half- life, etc. The moieties may alternatively decrease the toxicity of the molecule and eliminate or attenuate any undesirable side effect of the molecule, etc. Moieties capable of mediating such effects are disclosed in Remington's Pharmaceutical Sciences (1980). Procedure for coupling such moieties to a molecule  
20 are well known in the art.

Fragment. A "fragment" of a molecule such as Flt4 protein is meant to refer to any portion of the molecule, such as the peptide core, or a variant of the peptide core.

Variant. A "variant" of a molecule such as Flt4 protein is meant to refer  
25 to a molecule substantially similar in structure and biological activity to either the entire molecule, or to a fragment thereof. Thus, provided that two molecules possess a similar activity, they are considered variants as that term is used herein even if the composition or secondary, tertiary, or quaternary structure of one of the molecules is not identical to that found in the other, or if the sequence of amino acid residues is not  
30 identical.

Analogue. An "analogue" of Flt4 protein or genetic sequence is meant to refer to a protein or genetic sequence substantially similar in function to the Flt4 protein or genetic sequence herein.

#### DESCRIPTION OF PREFERRED EMBODIMENTS

5           The present invention is directed to what applicants have termed "Flt4,"  
a receptor for tyrosine kinase, Flt4-encoding nucleic acid molecules (e.g. cDNAs,  
genomic DNAs, RNAs, anti-sense RNAs, etc.), production of Flt4 peptides or Flt4  
protein from a *Flt4* gene sequence and its product, recombinant *Flt4* expression  
vectors, Flt4 analogues and derivatives, and diagnostic and/or therapeutic uses of Flt4  
10       and related proteins, Flt4 ligands, Flt4 antagonists and anti-Flt4 antibodies.

#### PRODUCTION OF RECOMBINANT Flt4.

Biologically active Flt4 may be produced by the cloning and expression  
of the Flt4-encoding sequence or its functional equivalent in a suitable host cell.

15       Production of Flt4 using recombinant DNA technology may be divided  
into a step-wise process for the purpose of description: (1) isolating or generating the  
coding sequence (gene) for the desired Flt4; (2) constructing an expression vector  
capable of directing the synthesis of the desired Flt4; (3) transfecting or transforming  
appropriate host cells capable of replicating and expressing the *Flt4* gene and/or  
processing the gene product to produce the desired Flt4; and (4) identifying and  
20       purifying the desired *Flt4* product.

#### ISOLATION OR GENERATION OF THE *Flt4* GENE

The nucleotide coding sequence of *Flt4* or functional equivalents  
thereof, may be used to construct recombinant expression vectors which will direct the  
expression of the desired Flt4 product. In the practice of the method of the invention,  
25       the nucleotide sequence depicted therein, or fragments or functional equivalents  
thereof, may be used to generate the recombinant molecules which will direct the  
expression of the recombinant Flt4 product in appropriate host cells. Flt4-encoding

nucleotide sequences may be obtained from a variety of cell sources which produce Flt4-like activities and/or which express Flt4-encoding mRNA. Applicants have identified a number of suitable human cell sources for Flt4, including human placenta, leukemia cells and some tumor cell lines.

5           The *Flt4* coding sequence may be obtained by cDNA cloning from RNA isolated and purified from such cell sources or by genomic cloning. The *Flt4* sequence may be for example amplified by polymerase chain reaction from cDNA or genomic DNA material using techniques well known in the art. Either cDNA or genomic libraries of clones may be prepared using techniques well known in the art and  
10   may be screened for particular *Flt4* DNAs with nucleotide probes which are substantially complementary to any portion of the *Flt4* gene. Full length clones, i.e., those containing the entire coding region of the desired *Flt4*, may be selected for constructing expression vectors. Alternatively, *Flt4* encoding DNAs may be synthesized in whole or in part by chemical synthesis using techniques standard in the  
15   art. Due to the inherent degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same or a functionally equivalent amino acid sequence may be used in the practice of the method of the invention. Such alterations of *Flt4* nucleotide sequences include deletions, additions or substitutions of different nucleotides resulting in a sequence that encodes the same or a functionally equivalent  
20   gene product. The gene product may contain deletions, additions or substitutions of amino acid residues within the sequence which result in silent changes thus producing a bioactive product. Such amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity and/or the amphipathic nature of the residues involved. For example, negatively charged amino  
25   acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; amino acids with uncharged polar head groups or nonpolar head groups having similar hydrophilicity values include the following: leucine, isoleucine, valine; glycine, alanine; asparagine, glutamine; serine, threonine; phenylalanine, tyrosine.



### CONSTRUCTION OF *Flt4* EXPRESSION VECTORS

Using this information, a variety of recombinant DNA vectors capable of providing the Flt4 receptor tyrosine kinase in reasonable quantities are provided. Additional recombinant DNA vectors of related structure that code for synthetic  
5 proteins having the key structural features identified herein as well as for proteins of the same family from other sources can be produced from the Flt4 receptor tyrosine kinase cDNA using standard techniques of recombinant DNA technology. A transformant expressing the Flt4 receptor tyrosine kinase has been produced as an example of this technology (see EXAMPLES 3 and 4). The newly discovered  
10 sequence and structure information can be used, through transfection of eukaryotic cells, to prepare the Flt4 receptor tyrosine kinase and its various domains for biological purposes.

### IDENTIFICATION OF TRANSFECTANTS OR TRANSFORMANTS EXPRESSING *Flt4* GENE PRODUCTS

15 The host cells which contain the recombinant coding sequence and which express the biologically active, mature product may be identified by at least four general approaches: (a) DNA-DNA, DNA-RNA or RNA-antisense RNA hybridization; (b) the presence or absence of "marker" gene functions; (c) assessing the level of transcription as measured by the expression of *Flt4* mRNA transcripts in the  
20 host cell; and (d) detection of the mature gene product as measured by immunoassay and, ultimately, by its biological activities.

In the first approach, the presence of *Flt4* coding sequences inserted into expression vectors may be detected by DNA-DNA hybridization using probes comprising nucleotide sequences that are homologous to the *Flt4* coding sequence.

25 In the second approach, the recombinant expression vector/host system may be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, resistance to methotrexate, transformation phenotype, occlusion body formation in baculovirus, etc.). For example, if the *Flt4* coding sequence is inserted within a marker gene

sequence of the vector, recombinants containing that coding sequence can be identified by the absence of the marker gene function. Alternatively, a marker gene can be placed in tandem with the *Flt4* sequence under the control of the same or different promoter used to control the expression of the *Flt4* coding sequence. Expression of the marker in response to induction or selection indicates expression of the *Flt4* coding sequence.

In the third approach, transcriptional activity for the *Flt4* coding region may be assessed by hybridization assays. For example, polyadenylated RNA can be isolated and analyzed by Northern blotting using a probe homologous to the *Flt4* coding sequence or particular portions thereof. Alternatively, total nucleic acids of the host cell may be extracted and assayed for hybridization to such probes.

In the fourth approach, the expression of Flt4 can be assessed immunologically, for example by Western blots, immunoassays such as radioimmunoprecipitation, enzyme-linked immunoassays and the like. The ultimate test of the success of the expression system, however, involves the detection of the biologically active *Flt4* gene product. Where the host cell secretes the gene product, the cell free media obtained from the cultured transfectant host cell may be assayed for Flt4 activity. Where the gene product is not secreted, cell lysates may be assayed for such activity. In either case, assays which measure ligand binding to Flt4 or other bioactivities of Flt4 may be used.

#### Flt4 DERIVATIVES, ANALOGUES AND PEPTIDES

The production and use of derivatives, analogues, and peptides related to Flt4 are also envisioned and are within the scope of the invention. Such derivatives, analogues, or peptides may have enhanced or diminished biological activities in comparison to native Flt4, depending on the particular application. Flt4 related derivatives, analogues, and peptides of the invention may be produced by a variety of means known in the art. Procedures and manipulations at the genetic and protein levels are within the scope of the invention. Peptide synthesis, which is standard in the art, may be used to obtain Flt4 peptides. At the protein level, numerous chemical

modifications may used to produce Flt4-like derivatives, analogues, or peptides by techniques known in the art, including but not limited to specific chemical cleavage by endopeptidases (e.g. cyanogen bromides, trypsin, chymotrypsin, V8 protease, and the like) or exopeptidases, acetylation, formylation, oxidation, etc.

5 Preferred derivatives, analogs, and peptides are those which retain Flt4 ligand binding activity. Those derivatives, analogs, and peptides which bind Flt4 ligand but do not transduce a signal in response thereto are useful as Flt4 inhibitors. Those derivatives, analogs, and peptides which bind Flt4 ligand and transduce a signal in response thereto, e.g., through a process involving intracellular Flt4  
10 autophosphorylation, are useful in the same manner as native Flt4. A preferred Flt4 ligand for use in such binding and/or autophosphorylation assays is a ligand comprising an approximately 23 kd polypeptide that is isolatable from a PC-3 conditioned medium as described herein. This ligand, designated Vascular Endothelial Growth Factor-C (VEGF-C), has been characterized in detail in PCT Patent Application  
15 PCT/FI96/00427, filed August 1, 1996, and published as International Publication WO 97/05250, and in the U.S. Patent Application priority documents relied upon therein for priority, all of which are incorporated herein by reference in their entirety.

#### ANTI-Flt4 ANTIBODIES

Also within the scope of the invention is the production of polyclonal  
20 and monoclonal antibodies which recognize Flt4 or related proteins.

Various procedures known in the art may be used for the production of polyclonal antibodies to epitopes of Flt4. For the production of antibodies, various host animals (including but not limited to rabbits, mice, rats, etc.) can be immunized by injection with Flt4, or a synthetic Flt4 peptide. Various adjuvants may be used to  
25 increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete) adjuvant, mineral gels such as aluminium hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially

useful human adjuvants such as BCG (*Bacillus Calmette-Guerin*) and *Corynebacterium parvum*.

5 A monoclonal antibody to an epitope of Flt4 may be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Köhler et al., *Nature*, 256: 495-497 (1975), and the more recent human B-cell hybridoma technique [Kosbor et al., *Immunology Today*, 4: 72 (1983)] and the EBV-hybridoma technique [Cole et al., *Monoclonal Antibodies and Cancer Therapy*, Alan R Liss, Inc., pp. 77-96 (1985)]. Antibodies against Flt4 also  
10 may be produced in bacteria from cloned immunoglobulin cDNAs. With the use of the recombinant phage antibody system it may be possible to quickly produce and select antibodies in bacterial cultures and to genetically manipulate their structure.

Antibody fragments which contain the idiotype of the molecule may be generated by known techniques. For example, such fragments include but are not  
15 limited to: the F(ab')<sub>2</sub> fragment which may be produced by pepsin digestion of the antibody molecule; the Fab' fragments which may be generated by reducing the disulfide bridges of the F(ab')<sub>2</sub> fragment, and the two Fab fragments which may be generated by treating the antibody molecule with papain and a reducing agent.

Antibodies to Flt4 may be used in the qualitative and quantitative  
20 detection of mature Flt4 and Flt4 precursor and subcomponent forms, in the affinity purification of Flt4 polypeptides, and in the elucidation of Flt4 biosynthesis, metabolism and function. Detection of Flt4 tyrosine kinase activity may be used as an enzymatic means of generating and amplifying a Flt4 specific signal in such assays. Antibodies to Flt4 may also be useful as diagnostic and therapeutic agents.

#### 25 USES OF Flt4, Flt4-ENCODING NUCLEIC ACID MOLECULES, AND ANTI-Flt4 ANTIBODIES

Applicants envision a wide variety of uses for the compositions of the present invention, including diagnostic and/or therapeutic uses of Flt4, Flt4 analogues

and derivatives, Flt4-encoding nucleic acid molecules, antisense nucleic acid molecules and anti-Flt4 antibodies.

Flt4-encoding nucleic acid molecules or fragments thereof may be used as probes to detect and quantify mRNAs encoding Flt4. Assays which utilize nucleic acid probes to detect sequences comprising all or part of a known gene sequence are well known in the art. *Flt4* mRNA levels may indicate emerging and/or existing neoplasias as well as the onset and/or progression of other human diseases. Therefore, assays which can detect and quantify *Flt4* mRNA may provide a valuable diagnostic tool.

Anti-sense *Flt4* RNA molecules are useful therapeutically to inhibit the translation of Flt4-encoding mRNAs where the therapeutic objective involves a desire to eliminate the presence of Flt4 or to downregulate its levels. *Flt4* anti-sense RNA, for example, could be useful as a Flt4 antagonizing agent in the treatment of diseases in which Flt4 is involved as a causative agent, for example due to its overexpression.

Additionally, *Flt4* anti-sense RNAs are useful in elucidating Flt4 functional mechanisms. Flt4-encoding nucleic acid molecules may be used for the production of recombinant Flt4 proteins and related molecules as separately discussed in this application.

Anti-Flt4 antibodies may be used to diagnose and quantify Flt4 in various contexts. For example, antibodies against various domains of Flt4 may be used as a basis for Flt4 immunoassays or immunohistochemical assessment of Flt4. Tyrosine kinase activity of Flt4 may be useful in these assays as an enzymatic amplification reaction for the generation of a Flt4 signal. Anti-Flt4 antibodies may also be useful in studying the amount of Flt4 on cell surfaces.

Antibodies may be produced which function as Flt4 ligand agonists or antagonists whereby the regulation of Flt4 activity becomes possible. Also, random peptides may be produced by synthetic means or by recombinant means from random oligonucleotides and the ones showing specific binding to the Flt4 receptor may be selected with the aid of the Flt4 extracellular domain. Such peptide segments also may be selected from a phage display library using the extracellular domain of Flt4, using

methods standard in the art. Such peptides may have agonistic or antagonistic activity. Flt4 antibodies may also provide valuable diagnostic tools after conjugation to various compounds for *in vivo* imaging of Flt4 expressing cells and tissues or tumors.

5 Monoclonal antibodies against Flt4 may be coupled either covalently or noncovalently to a suitable supramagnetic, paramagnetic, electron-dense, echogenic or radioactive agent to produce a targeted imaging agent. Antibody fragments generated by proteolysis or chemical treatments or molecules produced by using the epitope binding domains of the monoclonal antibodies could be substituted for the intact antibody. This imaging agent would then serve as a contrast reagent for X-ray, 10 magnetic resonance, sonographic or scintigraphic imaging of the human body for diagnostic purposes.

#### MOLECULAR BIOLOGY OF Flt4

The complete sequences of the *Flt4* cDNA clones set forth in SEQ ID NOs: 1 and 3 extend for 4195 or 4795 nucleotides and contain open reading frames of 15 1298 or 1363 amino acids, depending on alternative splicing. The nucleotide and deduced Flt4 amino acid sequence (short form) is shown in SEQ ID NOs: 1 and 2. Figure 2 depicts a comparison of the Flt4 amino acid sequence with that of the Flt1 tyrosine kinase amino acid sequence. See Shibuya et al., *Oncogene*, 5: 519-524 (1990).

20 A putative signal peptide sequence of mostly hydrophobic amino acids follows the initiator methionine. The sequence surrounding the corresponding ATG is in agreement with the consensus translation initiation sequence [Kozak, *Nucl. Acids Res.*, 15: 8125-8135 (1987)]. The predicted extracellular portion of both Flt4 polypeptides is 775 amino acids long and contains twelve potential sites for 25 asparagine-linked glycosylation (NXS/T). It also contains several amino acid residues exhibiting a pattern of spacing described for members of the immunoglobulin superfamily of proteins [Williams et al., *Annu. Rev. Immunol.*, 6: 381-405 (1988)]. It has 12 cysteine residues and it can be organized in seven immunoglobulin-like domains. The predicted Ig-like domain IV lacks cysteine residues. FIG. 2 also shows

the extracellular domain of Flt1 (SEQ. ID No. 5), which is the closest human homologue of Flt4. From this figure one can see the alignment of the cysteine residues and the very similar composition of the Ig-like regions.

The cytoplasmic domain of Flt4 is separated from the extracellular part  
5 by a putative transmembrane region of 23 hydrophobic amino acid residues. This sequence is flanked on the cytoplasmic side by a basic region suggesting the junction between the transmembrane and cytoplasmic domains. The tyrosine kinase homologous domain begins at residue 843 and includes an ATP-binding pocket and a putative autophosphorylation site homologous to Y416 of *c-src* at Y1068 (FIG. 2).  
10 The tyrosine kinase catalytic domain of Flt4 is divided into two subdomains by a 65 amino acid sequence (aa 944-1008) which is mostly hydrophilic and does not show homology to Flt1. Unlike Flt1, Flt4 does not contain tyrosine residues in its kinase insert.

A second species of *Flt4* mRNA has an alternative 3' end which  
15 encodes a longer form of the Flt4 protein.

In FIGS. 3A-C, production of short and long forms of the *Flt4* mRNA by alternative splicing is illustrated. FIG. 3A shows the schematic structure of the 3' ends of the cDNA inserts of clones J.1.1 and I.1.1. The TAG stop codon of clone J.1.1 as well as the polyadenylation site (polyA) are indicated. Clone I.1.1 differs from  
20 clone J.1.1 in the shaded segment (the long and short forms of *Flt4* mRNA, respectively). TAA and polyA indicate the stop codon and polyadenylation site of clone I.1.1. In addition, the restriction endonuclease cleavage sites for *EcoRI* and *AvaI* have been indicated. Shown below is the 256 bp *EcoRI-AvaI* insert of clone I.1.1. used for cRNA protection analysis. The most heavily-shaded segment indicates  
25 sequences from the polylinker in the linearized sense RNA template for transcription of the antisense strand *in vitro*. Also shown are the schematic structures of the protected fragments after RNase protection analysis. FIGS. 3B and 3C, show autoradiograms of the 256 bp <sup>32</sup>S-labeled antisense RNA probe and the 211 and 124 bp digested fragments representing the long and short forms of *Flt4* RNA when protected by  
30 polyadenylated RNA from the indicated cell lines (Tera-2 is a teratocarcinoma cell line,

which has been analyzed here with or without retinoic acid (RA) treatment for 10 days.) The (negative) control lane shows results of protection with transfer RNA. Note the downregulation of *Flt4* mRNAs during the differentiation of the Tera-2 cells. Tera-2 cells of clone 13 were provided by Dr. C.F. Graham (Department of Zoology, University of Oxford, UK). Cells between passages 18-40 were used in this study. The cells were maintained in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum and antibiotics. To induce differentiation, the cells were plated on gelatin-coated tissue-culture grade dishes at a density of  $1.5 \times 10^3$  cells/cm<sup>2</sup>. On the following day,  $2 \times 10^{-6}$  M RA was added to the medium. The cells were cultured in the presence of RA for up to 10 days.

Results shown in FIGS. 3A-C illustrate the generation of carboxy termini of these two *Flt4* (short and long) forms generated by alternative splicing.

According to its deduced amino acid sequence, Flt4 belongs to class III RTKs. More specifically, Flt4 belongs to a subfamily of RTKs, which contain seven Ig-loops in their extracellular part and thus it differs from other members of class III RTKs which contain five Ig-loops. *Flt4* is most closely homologous with the prototype receptor of the *FLT* family, *Flt1*, which was cloned as a *v-ros*-related DNA from a human genomic DNA library [Shibuya et al., *Oncogene*, 5: 519-524 (1990)] and with the mouse FLK1 receptor, which was cloned from hematopoietic stem cell-enriched fractions of mouse liver [Matthews et al., *Cell*, 65: 1143-1152 (1991); Matthews et al., *Proc. Natl. Acad. Sci. USA*, 88: 9026-9030 (1991)]. The extracellular domain of Flt4 shows 33% and 37% amino acid sequence identity with human Flt1 and mouse FLK1, respectively. *Flt1* and *FLK1*, like *Flt4*, are widely expressed in various normal tissues, such as lung, heart, and kidney. In addition, a recently identified human endothelial cell receptor tyrosine kinase KDR [Terman et al., *Oncogene*, 6: 1677-1683 (1991)] shows considerable homology with *Flt4* and *Flt1* family members. From the available sequence data one may calculate that KDR is 81% identical with Flt4 in the tyrosine kinase (TK) domain. In addition, the extracellular domain of KDR also has a seven Ig-loop structure and its TK1 and TK2 domains are



95% and 97% identical with the corresponding domains of mouse FLK1 receptor. This suggests that KDR is the human homologue of mouse FLK1.

While the *Flt4* TK domain is about 80% identical with the TK domains of *Flt1* and *FLK1/KDR*, it is only about 60% identical with the TK domains of other  
5 receptors of the RTK class III. As these other receptors also have only five Ig-like domains in the extracellular region, one can classify *Flt4*, *Flt1* and *FLK1/KDR* in a separate *FLT* subfamily within class III RTKs.

The tyrosine residue located in the sequence  
D/E-D/E-Y-M/V-P/D/E-M [Cantley, et al., *Cell*, 64: 281-302 (1991)] (SEQ. ID NO.  
10 6) in kinase inserts of PDGFRs, *c-fms* and *c-kit* is an autophosphorylation site, which, when phosphorylated, binds the SH2 domain of phosphatidylinositol 3'-kinase (PI-3K) [Reedijk et al., *EMBO J.*, 11: 1365-1372 (1992)]. Interestingly, unlike these class III RTKs, members of the *FLT* subfamily or the *Flt3/FLK2* receptor do not contain such consensus motifs.

15 The eight human class III RTK genes are clustered in three different chromosomes. Chromosome 4 contains the *c-kit*, PDGFR- $\alpha$  and KDR genes [Yarden et al., *EMBO J.*, 6: 3341-3351 (1987); Stenman et al., *Genes, Chromosomes, Cancer*, 1: 155-158 (1989); Terman et al., *Oncogene*, 6: 1677-1683 (1991)]. The *Flt1* and *Flt3* genes are located in chromosome 13q12 [Sato et al., *Jpn. J. Cancer Res.*, 78:  
20 772-775 (1987); Rosnet et al., *Genomics*, 9: 380-385 (1991)], while *Flt4* is localized in chromosome 5 band q35 [Aprelikova et al., *Cancer Res.*, 52: 746-748 (1992)]; close to the *fms* and PDGFR- $\beta$  genes [Warrington et al., *Genomics*, 11: 701-708 (1991)]. The long arm of chromosome 5 is involved in translocations found in leukemia cells. Deletions of part of the long arm of chromosome 5 were found in the bone  
25 marrow cells of patients with refractory anemia and macrocytosis [Van Den Berghe et al., *Nature*, 251: 437-439 (1974)]. An abnormal 5q chromosome is found in a few other myeloproliferative diseases, such as refractory anemia with excess blasts [Swolin et al., *Blood*, 58: 986-993 (1981)], agnogenic myeloid metaplasia [Whang-Peng et al., *Leuk. Res.*, 2: 41-48 (1978)], chronic myelogenous leukemia [Tomiyasu et al., *Cancer*  
30 *Genet. Cytogenet.*, 2: 309-315 (1980)], polycythemia vera [Van Den Berghe et al.,

*Cancer Genet. Cytogenet.*, 1: 157-162 (1979)] and essential thrombocythemia [Nowell et al., *Cancer*, 42: 2254-2260 (1978)].

The findings on *Flt4* mRNA expression suggest that its protein product is characteristic for certain leukemia cells. Several differentiation antigens shared  
5 between megakaryoblastic and endothelial cells have been shown to exist, one example being the platelet glycoprotein IIIa [Ylänne et al., *Blood*, 72: 1478-1486 (1988); Kieffer et al., *Blood*, 72: 1209-1215 (1988); Berridge et al., *Blood*, 66: 76-85 (1985)]. In addition, *Flt4* is expressed by certain endothelial cells of, e.g., the lung and kidney during the fetal period.

10 To further understand the role of *Flt4* during development, partial cDNAs for mouse *Flt4* were cloned. Using these probes in *in situ* hybridization, *Flt4* mRNA expression during mouse development was analyzed. It was determined that *Flt4* is expressed during vasculogenesis and angiogenesis of the lymphatic system. The relevance of these findings was also confirmed in normal and pathological human adult  
15 tissues, as *Flt4* was found in lymphatic endothelial cells of human adult tissues both in normal and pathological conditions, as well as in some high endothelial venules (HEVs).

The cloning of mouse *Flt4* cDNA fragments showed that their deduced amino acid sequence is almost identical with the corresponding human sequence  
20 (amino acid identity about 96 % in both segments studied). Further evidence for the identity of the mouse *Flt4* cDNA was obtained from Northern hybridization studies, wherein probes from both species yielded the typical 5.8 kb mRNA signal from mouse tissues. Analysis of RNA isolated from various tissues of adult mice showed *Flt4* expression in the liver, lung, heart, spleen and kidney, with no or very little  
25 hybridization in the brain and testes. This pattern is similar to the pattern reported earlier by Galland et al., *Oncogene*, 8: 1233 (1993). The results of RNase protection suggested that the *Flt4* gene is needed during mouse development, starting from 8.5 day p.c. embryos, and the relative expression levels appeared quite stable.

For the *in situ* hybridization, two fragments of mouse *Flt4* cDNA were  
30 selected which encode sequences of the extracellular domain. This allowed a clear

distinction of the hybridization pattern from the related FLK-1 and *Flt1* receptor patterns, which show only a very low degree of sequence identity with *Flt4* in the extracellular region. See Millauer *et al.*, *Cell*, 72: 835 (1993); Yamaguchi *et al.*, *Development*, 118:489 (1993); Peters *et al.*, *Proc. Natl. Acad. Sci. USA*, 90: 8915 (1993); Finnerty *et al.*, *Oncogene*, 8:: 2293 (1993).

*Flt4*, similar to the FLK-1, *Flt1*, *Tie* and *Tek* endothelial receptor tyrosine kinase genes, was not expressed in 7.5 day post-coitum (p.c.) embryos. In a 8.5-day p.c. embryo, the strongest *Flt4* signals were localised in the allantois, the angioblasts of head mesenchyme, the dorsal aortae, and the cardinal vein. Weak signals were seen in the endocardium. In contrast, angioblasts of the yolk sac were negative, unlike for FLK-1 and *Flt1*, *Tie* and *Tek*. See Korhonen *et al.*, *Oncogene*, 8: 395 (1993); and Peters *et al.*, *Proc. Natl. Acad. Sci. USA*, 90: 8915 (1993). The restriction of *Flt4* expression to the venous system was even more clear in samples from 11.5 day mouse embryos, where the *Tie* mRNA was expressed also in arteries.

In 12.5-day p.c. embryos the *Flt4* signal decorated developing venous and presumptive lymphatic endothelia, but unlike for the endothelial *Tie* receptor tyrosine kinase, arterial endothelia were negative. During later stages of development, *Flt4* mRNA became restricted to vascular plexuses devoid of blood cells, representing developing lymphatic vessels. Only the lymphatic endothelium and some high endothelial venules expressed *Flt4* mRNA in adult human tissues. Increased expression occurred in lymphatic sinuses and high endothelial venules, in metastatic lymph nodes, and in lymphangioma.

Due to difficulties in the interpretation of data from mouse embryos, human endothelia were studied, because the lymphatic system is much better defined in humans. Also, cells established from various endothelia could be studied in cell culture to see if the specificity of *Flt4* expression persists in *in vitro* conditions. Endothelial cells lines are known to lose differentiated features upon *in vitro* culture. Therefore, it was not unexpected that they were negative for *Flt4* mRNA. Cultured aortic endothelial cells were also devoid of *Flt4* mRNA. However, signals were obtained from human endothelial cells grown from the microvasculature and from femoral and

umbilical veins. Thus, at least some of the specificity of *Flt4* expression was retained in cell culture.

*In situ* hybridization analysis of adult human tissues confirmed the restriction of *Flt4* to the lymphatic system seen in the developing mouse embryos. *Flt4* expression was seen in the lymphatic endothelia and in the sinuses of human lymph nodes. Interestingly, also some of the HEVs, which have a cuboidal endothelium, shown to function in the trafficking of leukocytes to the lymph nodes, were *Flt4*-positive. Furthermore, a parallel hybridization analysis showed that *Flt4* mRNA levels were enhanced in these structures in metastatic as compared to normal lymph nodes. *Flt4* was also very prominent in lymphangiomas, which are benign tumours composed of connective tissue stroma and growing, endothelial-lined lymphatic channels. *Flt4* mRNA was restricted to the lymphatic endothelium of these tumors and absent from their arteries, veins and capillaries. In the human lung, lymphatic structures were the only *Flt4*-positive vessels identified.

The foregoing results indicate that *Flt4* is a novel marker for lymphatic vessels and some high endothelial venules in human adult tissues. The results also support the theory on the venous origin of lymphatic vessels. *Flt4*, as a growth factor receptor, may be involved in the differentiation and functions of these vessels. A detailed characterization of biological effects mediated through *Flt4* via the *Flt4* ligand, VEGF-C, is provided in PCT Patent Application PCT/FI96/00427, filed August 1, 1996, and published as International Publication WO 97/05250.

These results, combined with the *Flt4*-binding compounds according to the present invention, allows a selective labeling of lymphatic endothelium, especially by using antibodies of the present invention coupled to radioactive, electron-dense or other reporter substances, which can be visualized. It may be possible to inject into the lymphatic system substances, containing *Flt4* receptor internalization-inducing monoclonal antibodies or ligands, and thereby transport predefined molecules into the lymphatic endothelium. Also, it may be possible to use *Flt4*-binding compounds according to the invention for the detection of high endothelial venules, especially

activated HEVs, which express enhanced levels of the Flt4 receptor. To our knowledge, no such specific markers are currently available for lymphatic endothelium.

The following examples are given merely to illustrate the present invention and not in any way to limit its scope.

## EXAMPLE 1

### Isolation and characterization of cDNA clones encoding Flt4

#### MATERIALS AND METHODS

An oligo-dT primed human HEL cell cDNA library in bacteriophage  
10 lambda gt11 [A kind gift from Dr. Mortimer Poncz, Childrens Hospital of Philadelphia, PA; Poncz et al., *Blood*, 69: 219-223 (1987)] was screened with a cDNA fragment PCR-amplified from the same library [Aprelikova et al., *Cancer Res.*, 52: 746-748 (1992)]. Positive plaques were identified and purified as described [Sambrook et al.,  
15 *Molecular Cloning - A Laboratory Manual*, Cold Spring Harbor Laboratory Press, (1989)]. cDNA inserts of bacteriophage lambda were isolated as *Eco*RI fragments and subcloned into a GEM3Zf(+) plasmid (Promega). The entire Flt4 protein coding region was isolated. Three overlapping clones isolated from the HEL-library (as illustrated in Fig. 1) were sequenced using the dideoxy chain termination method with  
20 oligonucleotide primers designed according to the sequences obtained. All portions of the cDNAs were sequenced on both strands. Sequence analyses were performed using the GCG package programs [Devereux et al., *Nucleic Acids Res.*, 12: 387-395 (1984) and the Prosite program for Apple MacIntosh].

FIG. 1A illustrates a schematic structure of the *Flt4* cDNA clones analyzed. Arrows delineate subcloned restriction fragments (whose sizes are shown in  
25 kb) used for probing Northern blots depicted in Fig. 1B. E=*Eco*RI site, S=*Sph*I site. FIG. 1B illustrates Northern hybridization analysis of DAMI and HEL leukemia cell RNAs with the probes shown in Fig. 1A.

## RESULTS

A 210 bp long *Flt4* cDNA fragment isolated by a PCR cloning method from a HEL cell cDNA library was used as a molecular probe to screen an oligo-dT-primed human erythroleukemia cell cDNA library.

5                   Nucleotide sequence analysis of clones revealed an open reading frame of 1298 amino acid (aa) residues (SEQ ID NO: 2, FIG. 2). The translational initiator methionine marked in the figure is surrounded by a typical consensus sequence [Kozak, *Nucleic Acids Res.*, 12: 857-872 (1984)] and followed by a hydrophobic amino acid sequence characteristic of signal sequences for translocation into the  
10                   endoplasmic reticulum.

                  The extracellular domain of Flt4 can be aligned into seven immunoglobulin-like loops (FIG. 2). The figure also shows the comparison of Flt4 with Flt1, which contains very similar structures. The amino acid sequence of Flt1 is set forth as SEQ. ID NO: 5.

15                   Amino acid residues 775-798 form a hydrophobic stretch of sequence, which is likely to function as the transmembrane domain of the receptor, followed by several basic residues on the putative cytoplasmic side of the polypeptide. The juxtamembrane domain is 44 residues long before the beginning of a tyrosine kinase sequence homology at aa 842. With the interruption of homology in the kinase insert  
20                   sequence of 65 aa, this homology is first lost at 1175 aa at carboxyl terminal tail of the receptor. A search for related tyrosine kinase domains in the amino acid sequence database (Swissprot and NBRF) identifies the Flt1 and PDGFRB tyrosine kinases with homology of about 80 and 60% in the catalytic tyrosine kinase regions respectively.

## EXAMPLE 2

### 25                   Preparation of an anti-Flt4 antisera

                  A 657 base pair *EcoRI* fragment encoding the predicted C-terminus of Flt4 short form was cloned in-frame with the glutathione-S-transferase coding region in the pGEX-1 $\lambda$ T bacterial expression vector (Pharmacia) to produce a GST-Flt4 fusion protein in *E. coli*. The resulting fusion protein was produced in bacteria and

partially purified by glutathione affinity chromatography according to the manufacturer's instructions. This protein was used in immunization of rabbits in order to produce polyclonal antibodies against Flt4. Antisera were used after the third booster immunization.

5

### EXAMPLE 3

#### Expression of Flt4 in COS cells

#### MATERIALS AND METHODS

The full-length Flt4 protein coding sequence (combined from three clones, FIG. 1) was inserted into the *HindIII-BamHI* site of SVpoly mammalian expression vector [Stacey et al., *Nucleic Acids Res.*, 18: 2829 (1990)] construct SV14-2. The expression vectors (SV-FLT4 short and SV-FLT4 long, containing the respective forms of *Flt4* cDNA) were introduced into COS cells by DEAE-dextran transfection method [McCutchan et al., *J. Natl. Cancer Inst.*, 41: 351-357 (1968)].

Two days after transfection, the cells were washed with phosphate-buffered saline (PBS) and scraped into immunoprecipitation buffer (10mM Tris pH 7.5, 50 mM NaCl, 0.5% sodium deoxycholate, 0.5% Nonidet P40, 0.1% SDS, 0.1 TIU/ml Aprotinin). The lysates were sonicated, centrifuged for 15' at 10,000 x g and incubated overnight on ice with 3 ml of the antisera. Protein A sepharose (Pharmacia) was added and the incubation was continued for 30' with rotation. The precipitates were washed four times with the immunoprecipitation buffer, once with PBS and once with aqua before analysis in SDS-PAGE.

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15

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#### RESULTS

The structural predictions of the *Flt4* cDNA sequence were tested by cloning the full-length *Flt4* short and long protein-coding regions into the *HindIII-BamHI* sites of the pSVpoly expression vector and transfecting these expression vectors into COS cells. The proteins produced by these two constructs differ in their C-terminus: the longer form contains an additional 65 amino acids. Two days after transfection, the cells were lysed and immunoprecipitated using antibodies

25

generated against the GST-Flt4 fusion protein containing 40 carboxyl terminal amino acid residues of the short form of the predicted Flt4 protein (i.e., a portion common to both the short and long forms of *Flt4*). Immunoprecipitated polypeptides were analyzed by SDS-polyacrylamide gel electrophoresis. The preimmune serum did not  
5 reveal any specific bands, whereas the Flt4-specific antibodies recognize two bands of about 170 and 190 KD. These two bands may represent differentially glycosylated forms of Flt4 protein.

#### EXAMPLE 4

##### Expression of Flt4 in NIH3T3 cells

##### MATERIALS AND METHODS

The full-length *Flt4* cDNA (short form) was subcloned into the LTRpoly vector (see Makela, *et al.*, *Gene*, 118:293-294 (1992), disclosing plasmid vector pLTRpoly, having ATCC accession number 77109 and GeneBank accession number X60280) containing the Moloney murine leukemia virus long terminal repeat  
15 promoter. This LTR-FLT4 expression vector was used with pSV2neo marker plasmid to co-transfect NIH3T3 cells, and G418 resistant clones were analyzed for Flt4 expression.

For Western immunoblotting analyses, cells on one confluent large plate were lysed in 2.5 % SDS, 125 mM Tris, pH 6.5. Cell lysates were electrophoresed on  
20 SDS-page and electroblotted onto a nitrocellulose membrane. The membrane was incubated with the antiserum raised against the Flt4 carboxy-terminus peptide, and bound antibodies were visualized using horseradish peroxidase conjugated swine anti-rabbit antiserum (Dako) and ECL reagents (Amersham). For metabolic labeling, the cultures were labeled with 100 $\mu$ Ci/ml  $^{35}$ S-methionine for one hour. After labelling,  
25 cells were washed twice and incubated in their growth medium for 1 or 2 hours, lysed, immunoprecipitated with anti-Flt4 antibodies, and analyzed by SDS-PAGE and autofluorography.



## RESULTS

The 170 and 190 KD polypeptides could be detected in the *Flt4* short form-transfected into NIH3T3 cells, but not in cells transfected with pSV2neo only. In addition to these two bands, a major band of about 120 Kd was observed in the clones  
5 producing Flt4. Metabolic labeling and pulse-chase experiments showed that this protein is generated as a result of post-translational processing of the short form Flt4 polypeptides.

### EXAMPLE 5

#### Chromosomal mapping of the *Flt4* locus

10 Because some clustering of class III receptor genes has been observed, it is of great interest to determine the chromosomal localization of *Flt4*. Thus, rodent-human cell hybrids were analyzed, indicating linkage of *Flt4* to human chromosome 5.

Localization of the *Flt4* gene in the region 5q33->5qter was determined  
15 using hybrids carrying partial chromosome 5s. These hybrids were tested for presence of the *Flt4* locus by filter hybridization. The region of chromosome 5 common to *Flt4*-positive hybrids and absent from the *Flt4*-negative hybrids was 5q33.1-qter. The presence of human chromosome 5q33-qter in the hybrids is thus correlated with the presence of *Flt4* sequences. The regional mapping results indicated that the *Flt4* locus  
20 is telomeric to the CSF1R/platelet-derived growth factor receptor- $\beta$  (PDGFRB) locus as well as to the  $\beta$ -adrenergic receptor (ADRBR) locus since these loci are all present in the hybrid GB13, which was negative for *Flt4*.

### EXAMPLE 6

#### Expression of the *Flt4* mRNA in tumor cell lines and endothelial cells

25 The leukemia cell lines (K562) used in this study have been reported in several previous publications; [Lozzio et al., *Blood*, 45: 321-334 (1975)], HL-60 [Collins et al., *Nature*, 270: 347-349 (1977)], HEL [Martin et al., *Science*, 216:

1233-1235 (1982)], DAMI [Greenberg et al., *Blood*, 72: 1968-1977 (1988)], MOLT-4 [Minowada et al., *J. Natl. Cancer Inst.*, 49: 891-895 (1972)], Jurkat [Schwenk et al., *Blut*, 31: 299-306 (1975)], U937 [Sundström et al., *Int. J. Cancer*, 17: 565-577 (1976)], KG-1 [Koeffler et al., *Science*, 200: 1153-1154 (1978)], JOK-1 [Andersson et al., 1982, in R. F. Revoltella (ed.), *Expression of Differentiated Functions in Cancer Cells*, 239-245, Raven Press, New York] and ML-2 [Gahmberg et al., 1985, in L. C. Andersson, et al. (ed.), *Gene Expression During Normal and Malignant Differentiation*, 107-123, Academic Press, London]. The following tumor cell lines, obtained from the American Type Culture Collection also were analyzed: JEG-3, a choriocarcinoma; A204, a rhabdomyosarcoma; SK-NEP-1, a nephroblastoma; BT-474, a breast carcinoma; Y79, a retinoblastoma. The leukemia cells were grown in RPMI containing 10% fetal calf serum (FCS) and antibiotics. Dami cells were cultivated in Iscove's modified DMEM with 10% horse serum. A permanent endothelial hybrid cell line (EAhy926) obtained by fusing first-passage human umbilical vein endothelial cells with the A549 lung carcinoma cells [Edgell et al., *Proc. Natl. Acad. Sci. USA*, 50: 3734-3737 (1983)] was cultured in DMEM-HAT medium containing 10% FCS and antibiotics.

Poly(A)<sup>+</sup> RNA was extracted from the cell lines as described [Sambrook et al., see above]. 5 µg of the Poly(A)<sup>+</sup> RNA samples were electrophoresed in agarose gels containing formaldehyde and blotted using standard conditions [Sambrook et al., see above]. The inserts of the *Flt4* cDNA clones were labelled by the random priming method and hybridized to the blots. Hybridization was carried out in 50% formamide, 5x Denhardt's solution (100x Denhardt's solution is 2% each of Ficoll, polyvinylpyrrolidone and bovine serum albumin), 5 x SSPE (3M NaCl, 200 mM NaH<sub>2</sub>PO<sub>4</sub> · H<sub>2</sub>O, 20 mM EDTA, pH 7.0), 0.1% SDS (sodium dodecyl sulphate), and 0.1 mg/ml of sonicated salmon sperm DNA at 42°C for 18-24 h. The filters were washed at 65°C in 1x SSC (150 mM NaCl, 15 mM sodium citrate, pH 7.0), 0.1% SDS and exposed to Kodak XAR-5 film.

Northern analyses were performed with the extracted poly(A)<sup>+</sup> RNA from eight leukemia cell lines (HEL, K562, DAMI, U937, MOLT4, HL60, Jurkat, and

KG-1) and the endothelial hybrid cell line (EAhy926). Hybridization with the GAPDH probe was used as an internal control for the loading of even amounts of RNA to the analysis. Only the HEL erythroleukemia cells, and DAMI megakaryoblastic leukemia cells expressed 5.8 kb and 4.5 kb *Flt4* mRNA. The K562 erythroleukemia, Jurkat and MOLT-4 T-cell leukemias, as well as HL-60 promyelocytic leukemia, U937 monocytic leukemia, and KG-1 myeloid leukemia cells were negative for the *Flt4* mRNA.

Northern analyses were performed with the extracted poly(A)<sup>+</sup> RNA from five tumor cell lines (JEG-3, A-204, SK-NEP-1, BT-474, and Y79) and two of the aforementioned leukemia cell lines (JOK-1, MOLT4). The labeled S2.5 cDNA clone (see Fig. 1) was used as the hybridization probe. Hybridization with a  $\beta$ -actin probe was used as an internal control for the loading of even amounts of RNA to the analysis. Only the SK-NEP-1 neuroblastoma and Y79 retinoblastoma cells were observed to contain *Flt4* transcripts.

Tera-2 teratocarcinoma cells were analyzed after a 10 day treatment with vehicle (-) or retinoic acid (+) to induce neuronal differentiation [Thompson et al., *J. Cell Sci.*, 72: 37-64 (1984). In Northern blotting analysis of poly(A)<sup>+</sup> RNA isolated from the cells it was found that the undifferentiated cells expressed 5.8 kb and 4.7 kb mRNAs for *Flt4*, but after the 10 day differentiation, no *Flt4* mRNA could be detected in Northern blotting and hybridization. These results indicate that *Flt4* was downregulated during the differentiation of these cells.

*Flt4* mRNA expression also was analyzed in undifferentiated and TPA-differentiated HEL cells. Both the HEL and DAMI cell lines possess a dual erythroid/megakaryoblastic phenotype and can be induced to further expression of megakaryoblastic markers by treatment with the tumor promotor 12-O-tetradecanoylphorbol-13-acetate (TPA). We analyzed whether *Flt4* expression is stimulated in these cells during their differentiation. HEL cells were analyzed 2 days after treatment with TPA or with DMSO used to dissolve it. After stripping off the *Flt4* signal, the filter was probed with Rb-1 and  $\beta$ -actin cDNAs to confirm an even loading of the lanes. On the basis of densitometric scanning analysis of the autoradiograph and normalization against the constitutive expression of the GAPDH

gene, it was determined that the *Flt4* mRNA level was increased about 3.4 fold in TPA-induced HEL cells, when the cells undergo megakaryoblastic differentiation.

## EXAMPLE 7

### Expression of *Flt4* in fetal lung

5                    *In situ* hybridization: Lung tissue from a 15 week-old human fetus was obtained with the permission of joint ethical committee of the University Central Hospital and the University of Turku, Finland. The sample was fixed in 10% formalin for 18 hours at 4°C, dehydrated, embedded in wax, and cut into 6  $\mu$ m sections. The RNA probes of 206 and 157 bases (antisense and sense) were generated from  
10 linearized plasmid DNA using SP6 and T7 polymerases and [<sup>35</sup>S]-UTP. *In situ* hybridization of sections was performed according to Wilkinson et al., *Development*, 99:493-500 (1987); Wilkinson, *Cell*, 50:79-88 (1987), with the following modifications: 1) instead of toluene, xylene was used before embedding in paraffin wax; 2) 6 $\mu$ m sections were cut, placed on a layer of diethyl pyrocarbonate-treated  
15 water on the surface of glass slides pretreated with 2% 3-aminopropyl-triethoxysilane (Sigma); 3) alkaline hydrolysis of the probes was omitted; 4) the hybridization mixture contained 60 % deionized formamide; 5) the high stringency wash was for 80 minutes at 65°C in a solution containing 50 mM DTT and 1 x SSC; 6) the sections were covered with NTB-2 emulsion (Kodak) and stored at 4°C. After an exposure time of  
20 14 days, the slides were developed for 2.5 minutes in a Kodak D-19 developer and fixed for 5 minutes with Unifix (Kodak). The sections were stained with hematoxylin in water.

                    In the hybridization studies using the anti-sense probe, *Flt4* mRNA was observed mainly in certain endothelial cells of the lungs of a 15 week old fetus.

25                    Control hybridizations with the sense strand probe and with RNase A-treated sections did not give a signal above background.

                    For immunoperoxidase staining, a 1:100 dilution of the anti-*Flt4* antibody, peroxidase-conjugated swine anti-rabbit antibodies and methods standard in the art were used. Control stainings with preimmune serum or immunogen-blocked

serum did not give a signal. Lung tissue from seventeen-week old human fetuses were analyzed, and the results were consistent with those of the mRNA *in situ* hybridization experiments: the endothelium of certain large vessels of the lung were stained positive with the rabbit anti-Flt4 antiserum.

## EXAMPLE 8

### Identification of *Flt4* genes in non-human mammalian species

In FIG. 4 the results of an experiment examining the presence of *Flt4* sequences in DNA from different species is shown. In order to reveal how well the *Flt4* gene has been conserved in evolution, the 2.5 kb cDNA fragment (see FIG. 1) was hybridized to genomic DNAs purified from different animals and from yeast and digested with *EcoR*I. The hybridization solution comprised 50% formamide, 5x Denhardt's solution, (100x Denhardt's solution is 2% each of Ficoll, polyvinylpyrrolidone and bovine serum albumin), 5x saline-sodium phosphate-EDTA (3M NaCl, 200 mM NaH<sub>2</sub>PO<sub>4</sub>-H<sub>2</sub>O, 20 mM EDTA, pH 7.0), 0.1% sodium dodecyl sulfate, and 0.1 mg/ml sonicated salmon sperm DNA. Hybridization was performed at 42°C for 24 hours. The filter was washed at 65°C in 1x standard saline citrate (150 mM NaCl, 15 mM sodium citrate, pH 7.0) and 0.1% sodium dodecyl sulfate and exposed to Kodak XAR-5 film. Specific bands were found in monkey, rat, mouse, dog, cow, rabbit, and chick DNAs, but the yeast DNA did not give a signal. The *Flt4* cDNA has been isolated from quails. See Eichmann *et al.*, *Gene*, 174(1): 3-8 (September 26, 1996) and Genbank accession number X83287.

## EXAMPLE 9

### *Flt4* gene expression in adult human tissues

*Flt4* mRNA expression in adult human tissues was analyzed using 2 µg of poly(A)<sup>+</sup> RNA from heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas tissues (Multiple Tissue Northern Blot, Clontech Inc.) by hybridization with

the *Flt4* cDNA probe. Control hybridizations with probes for constitutively expressed genes showed an even loading of the lanes.

Hybridization of poly(A)<sup>+</sup> RNA from various human tissues with the *Flt4* cDNA fragment showed mRNA bands of 5.8 and 4.5 kb mobility and a weakly labeled band of 6.2 kb in placenta, lung, heart and kidney. Faint mRNA bands were seen in the liver and skeletal muscle, whereas the pancreas and brain appeared to contain very little if any *Flt4* RNA.

#### EXAMPLE 10

##### *Flt4* expression in human fetal tissues

To examine *Flt4* mRNA expression in human fetal tissues, a Northern blot containing total RNA from the below-listed tissues of 16-19 week human fetuses was hybridized with the 1.9 kb *Flt4* cDNA fragment (see Fig. 1) and the resulting autoradiograph was scanned with a densitometer. The results were normalized for the amount of RNA estimated from a UV picture of the corresponding ethidium bromide (EtBr) stained gel. The following symbols denote mRNA levels in an increasing order: -, +, ++, +++.

TABLE 1

	<u>Fetal tissue</u>	<u>mRNA</u>
	Brain	
	Meninges	+
5	Cortical plate	++
	Intermediate zone	+++
	Ependymal zone	+
	Cerebellum	++
	Choroid plexus	+
10	Liver +	
	Pancreas	+
	Small intestine	-
	Heart +	
	Lung	+++
15	Kidney	++
	Adrenal	++
	Skin	++
	Spleen +++	
	Thymus	-

20                      Analysis of human fetal tissues showed that all except the thymus and small intestine contain *Flt4* transcripts. The highest expression levels were found in lung and spleen.

#### EXAMPLE 11

##### *Flt4* expression vector

25                      Full-length *Flt4* cDNA (short form) was produced by a) ligation of a *Sph*I-cleaved *Flt4* PCR fragment [amplified from the S2.5 kb clone (see FIG. 1) using

the primer oligonucleotides 5'-ACATGCATGC CACCATGCAG CGGGGCGCCG  
CGCTGTGCCT GCGACTGTGG CTCTGCCTGG GACTCCTGGA-3'(SEQ. ID NO.  
7) (forward) and 5'-ACATGCATGC CCCGCCGGT CATCC-3' (reverse)] (SEQ. ID  
NO. 8) to the 5' end of the S2.5 kb fragment, subcloned into the pSP73 vector  
5 (Promega), using two *Sph*I sites; b) ligation of a PCR fragment containing the last 138  
bps amplified from the 0.6 kb *Eco*RI fragment (see FIG. 1) with the oligonucleotide  
primers 5'-CGGAATTCCC CATGACCCCA AC-3'(SEQ. ID NO. 9) (forward) and  
5'-CCATCGATGG ATCCTACCTG AAGCCGCTTT CTT-3' (SEQ. ID NO. 10)  
(reverse) to the 3' end of construct a) using the *Eco*RI and *Bam*HI sites; c) ligation of a  
10 1.2 kb *Eco*RI fragment in the *Eco*RI site of construct b); d) ligation of the resulting full  
length *Hind*III-*Bam*HI fragment into the *Hind*III-*Bam*HI cleaved SV-poly expression  
vector [Stacey et al., *Nucl. Acids Res.*, 18: 2829 (1990)].

## EXAMPLE 12

### Identification of an Flt4 ligand

15               Conditioned media from the PC-3 prostatic adenocarcinoma cell line  
(ATCC CRL 1435) cultured for 7 days in F12 medium in the absence of fetal bovine  
serum (FBS) was cleared by centrifugation at 16 000 x g for 20 minutes and screened  
for the ability to induce tyrosine phosphorylation of Flt4.

                  NIH3T3-cells recombinantly expressing Flt4 (see Example 13) were  
20               reseeded on 5 cm diameter cell culture dishes and grown to confluency in Dulbecco's  
modified minimal essential medium (DMEM) containing 10% fetal bovine serum and  
antibiotics. The confluent cells were washed twice in phosphate-buffered saline (PBS)  
and starved in DMEM/0.2% bovine serum albumin overnight. For stimulation, the  
starvation medium was replaced by 1 ml of the conditioned medium and the cells were  
25               incubated at 37°C for 5 minutes.

                  After stimulation with the PC-3 conditioned medium, the culture plates  
containing the cells were put on ice and washed twice with Tris-HCl, pH 7.4, 150 mM  
NaCl containing 100 mM NaVO<sub>4</sub>. The washing solution was removed from the dishes  
and the cells were lysed in RIPA buffer [10 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.5%



sodium deoxycholate, 0.5% Nonidet P40, 0.1% sodium dodecyl sulphate (SDS)] containing aprotinin, 1 mM PMSF and 1 mM  $\text{NaVO}_4$ , and the lysates were sonicated for 10 seconds twice. The lysates were then centrifuged at  $16,000 \times g$  for 30 minutes and the supernatants were transferred to new tubes and used for immunoprecipitation.

5                   The polyclonal antibodies against the Flt4 C-terminus (described above) were used for immunoprecipitation. Supernatants from the cell lysates were incubated for 2 hours on ice with 2 to 4  $\mu\text{l}$  of rabbit polyclonal anti-Flt4 antiserum. About 30  $\mu\text{l}$  of a 50 % (vol/vol) solution of protein A-Sepharose (Pharmacia) in PBS was added, and incubation was continued for 45 minutes with rotation at  $+4^\circ\text{C}$ . The  
10 immunoprecipitates were washed three times with the RIPA buffer and once with PBS.

                  The immunoprecipitates were then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a 7.5 % gel and blotted on nitrocellulose. These Western blots were incubated with monoclonal anti-phosphotyrosine (anti-P-Tyr) antibodies (1:2000 dilution of PT-66 Sigma, cat. P-3300) followed by detection with  
15 peroxidase-conjugated rabbit anti-mouse antibodies (1:1000 dilution, Dako, cat. P 161) using the chemiluminescence detection system (Amersham). In some cases, the blots were stripped to clear previous signals for 30 minutes at  $50^\circ\text{C}$  in 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7 with occasional agitation and re-stained with anti-Flt4 antibodies (1:1000 dilution) followed by staining with  
20 peroxidase-conjugated swine anti-rabbit antibodies (1:1000 dilution, Dako, P217). As a positive control for the tyrosine phosphorylation of Flt4, anti-Flt4 immunoprecipitates from the Flt4-expressing NIH3T3 cells treated with 100 mM of the tyrosyl phosphatase inhibitor sodium pervanadate ( $\text{PerVO}_4$ ) for 20 minutes were used. Treatment of cells with Sodium pervanadate was done by addition of 100 mM  
25 (final concentration) of sodium orthovanadate and 2 mM (final concentration) of hydrogen peroxide to the cell medium and incubation of the cells for 20 minutes at  $37^\circ\text{C}$  5%  $\text{CO}_2$ . That procedure resulted in the generation of the peroxidized form of vanadate (vanadyl hydroperoxide), which is a very potent inhibitor of the protein tyrosine phosphatases in living cells.

The PC-3 cell conditioned medium stimulated tyrosine phosphorylation of a 120 kD polypeptide which co-migrated with tyrosine phosphorylated, processed mature form of Flt4. Co-migration was confirmed after restaining of the blot with anti-Flt4 antibodies.

5                   To prove that 120 kD polypeptide is not a non-specific component of the conditioned medium, 15 ml of conditioned medium were separated by SDS-PAGE, blotted on nitrocellulose, and the blot was stained with anti-P-Tyr antibodies. Several polypeptides were detected, but none of them comigrated with Flt4, indicating that the 120 kD band is indeed tyrosine-phosphorylated protein immunoprecipitated from the  
10                   stimulated cells. Analysis of stimulation by PC-3 conditioned medium pretreated with heparin Sepharose CL-6B (Pharmacia) for 2 hours at room temperature (lane 3) shows that the Flt4 ligand does not bind to heparin.

                  Unconditioned medium did not induce Flt4 autophosphorylation. Also, neither non-transfected NIH3T3 cells nor NIH3T3 cells transfected with the FGFR-4  
15                   showed tyrosine phosphorylation of the 120 kD polypeptide upon stimulation with the conditioned medium from PC-3 cells. Stimulating activity was considerably increased when the PC-3 conditioned medium was concentrated fourfold using a Centricon-10 concentrator (Amicon). Also, the flow through obtained after the concentration, containing proteins of less than 10,000 molecular weight (<10,000), did not stimulate  
20                   phosphorylation of Flt4. Pretreatment of the concentrated conditioned medium of PC-3 cells with 50 ml of the Flt4 extracellular domain (Flt4EC-6xHis, see below) coupled to CNBr-activated Sepharose (1 mg/ml) according to the manufacturer's instructions completely abolished the tyrosine phosphorylation of Flt4. Analogous pretreatment of the conditioned medium with Sepharose CL-4B did not affect its  
25                   stimulatory activity.

                  These data prove that PC-3 cells produce soluble ligand for Flt4. The above experiments prove that the ligand binds to the recombinant Flt4 EC domain. Thus, that ligand can be purified using the recombinant Flt4 EC domain in affinity chromatography. The purified protein can be electrophoresed in SDS-PAGE, blotted  
30                   onto polyvinylidene difluoride (PVDF) membranes and its amino terminal sequence can

be determined by methods standard in the art. Alternatively, the purified ligand can be digested to peptides for their amino terminal sequence determination. Peptide sequences obtained from the purified protein are used for the synthesis of a mixture of oligonucleotides encoding such sequences. Such oligonucleotides and their  
5 complementary DNA strand counterparts can be radioactively labelled by and used for the screening of cDNA libraries made from the PC-3 cells to obtain a cDNA encoding the ligand; all by methods standard in the art (Wen *et al.*, *Cell* 69: 559-572 (1992)). Alternatively, such oligonucleotides and their counterparts can be used as primers in polymerase chain reaction (PCR) to amplify sequences encoding the ligand using  
10 cDNA made from PC-3 cell RNA as a template. Such method of cDNA synthesis and PCR (RT-PCR) is standard in the art (Innis *et al.*, 1990, PCR protocols, Academic Press; McPherson, M.J. *et al.*, 1991, PCR, a practical approach, IRL Press; Partanen *et al.*, *Proc. Natl. Acad. Sci., USA*, 87: 8913-8917 (1990)). Yet another alternative is to clone the Flt4 ligand from the PC-3 cells by using cDNAs cloned into eukaryotic  
15 expression vector (*e.g.* using the Invitrogen Librarian cloning kit and vectors provided, such as pcDNA I or pcDNA III) and screening of such libraries transfected into, *e.g.*, COS cells with Flt4-alkaline phosphatase (Cheng and Flanagan, *Cell*, 79: 157-168, (1994)), Flt4-immunoglobulin (Flt4-Ig) (Lyman *et al.*, *Cell*, 75: 1157-1167 (1993)), or similar affinity reagents, by methods standard in the art.

20

### EXAMPLE 13

#### Cell lines and transfections.

NIH3T3 cells and 293-EBNA cells (Invitrogen) were cultured in DMEM containing 10% FCS. For stable expression, NIH3T3 cells were transfected with the LTR-FLT4l vector together with the pSV-neo vector (see Example 4, above)  
25 where the *Flt4* cDNA is expressed under the control of the Moloney murine leukemia virus LTR promoter, by the lipofection method using the DOTAP transfection reagent (Boehringer-Mannheim). COS-1 cells were transfected by the DEAE dextran method (McClutchan and Pagano, *J. Natl. Cancer Inst.*, 41: 351-35 (1968)). Transfected cells were selected in 500 mg/ml neomycin.

#### EXAMPLE 14

##### Construction and expression of Flt4 fusion proteins

The pVTBac-FLT4EC-6xHis fusion construct. The ends of cDNA encoding Flt4 were modified as follows: The 3' end of *Flt4* cDNA sequence encoding

5 the extracellular domain (EC) was amplified using oligonucleotides

5'-CTGGAGTCGACTTGGCGGACT-3' (SEQ ID NO: 13, *SalI* site underlined, containing sequence corresponding to nucleotides 2184-2204 of SEQ ID NO: 1) and 5'CGCGGATCCCTAGTGATGGTG

ATGGTGATGTCTACCTTCGATCATGCTGCCCTTATCCTC-3' (SEQ ID NO: 14,

10 *BamHI* site underlined, containing sequence complementary to nucleotides 2341-2324 of SEQ ID NO: 1) encoding 6 histidine residues for binding to a Ni-NTA column (Qiagen, Hilden, Germany) followed by a stop codon. The amplified fragment was

digested with *SalI* and *BamHI* and ligated as a *SalI*-*BamHI* fragment into the LTR-FLT4l vector (see Example 4), replacing a unique *SalI*-*BamHI* fragment containing

15 sequences encoding the Flt4 transmembrane and cytoplasmic domains.

The 5' end of the *Flt4* cDNA without the Flt4 signal sequence encoding region was amplified by PCR using oligonucleotides

5'-CCCAAGCTTGGATCCAAGTGGCTACTCCATGACC-3' (SEQ ID NO: 11, *HindIII* and *BamHI* sites underlined, containing sequence corresponding to nucleotides 20 86-103 of SEQ ID NO: 1) and 5'-GTTGCCTGTGATGTGCACCA-3' (SEQ ID NO: 12, containing sequence complementary to nucleotides 700-681 of SEQ ID NO: 1).

This amplified fragment (which included nucleotides 86-700 of SEQ ID NO: 1) was digested with *HindIII* and *SphI* (the *SphI* site, corresponding to nucleotides 588-593 of SEQ ID NO: 1, being within the amplified region of the *Flt4* cDNA).

25 The resultant *HindIII*-*SphI* fragment was used to replace a *HindIII*-*SphI* fragment in the modified LTR-FLT4l vector described immediately above (the *HindIII* site is in the 5' junction of the *Flt4* insert with the pLTRpoly portion of the vector, the *SphI* site is in the *Flt4* cDNA and corresponds to nucleotides 588-593 of SEQ ID NO: 1). The resultant Flt4EC-6xHis insert was then ligated as a *BamHI* 30 fragment into the *BamHI* site in the pVTBac plasmid (Tessier *et al.*, *Gene* 98: 177-183

(1991)). The construct was transfected together with baculovirus genomic DNA into SF-9 cells by lipofection. Recombinant virus was generated and used for infection of High-Five cells (Invitrogen).

5                   **The *Flt4*-AP fusion construct.** The 3' end of the sequence encoding the Flt4 EC domain was amplified using oligonucleotides 5'-CTGGAGTCGACTTGGCGGACT-3' (SEQ ID NO: 15) and 5'-CGGGATCCCTCCATGCTGCCCTTATCCT-3' (SEQ ID NO: 16) and ligated as *SalI*-*Bam*HI fragment into the LTR-FLT4l vector, replacing sequences encoding the transmembrane and cytoplasmic domains. The resulting insert was then ligated as a *Hind*III-*Bam*HI fragment into the *Hind*III-*Bgl*II sites of plasmid APTag-1 in frame with the alkaline phosphatase coding region (Flanagan and Leder, 1990, Cell 63, 185-194). NIH3T3 cells were co-transfected with this Flt4-AP construct and pSV2neo (Southern and Berg, *J. Mol. Appl. Genet.* 1: 327-341 (1982)) by lipofection using the DOTAP transfection reagent (Boehringer) and the transfected cells were selected in the presence of 500 mg/ml neomycin. The recombinant protein produced into the medium was detected by a colorimetric reaction for staining for alkaline phosphatase activity (Cheng and Flanagan, *Cell* 79: 157-168 (1994)).

20                   **The *Flt4*-Ig construct.** A recombinant DNA encoding an Flt4-immunoglobulin chimera was constructed as follows. The 5' end of the cDNA encoding Flt4, including *Flt4* nucleotides encoding the signal sequence, was amplified by PCR using primers 5'-GGCAAGCTTGAATTCGCCACCATGCAGCGGGGCGCC-3' (SEQ ID NO: 17) and 5'-GTTGCCTGTGATGTGCACCA-3' (SEQ ID NO: 18) and ligated as *Hind*III-*Sph*I fragment into the LTR-FLT4l vector. The 3' end of Flt4 EC-encoding sequence was amplified using oligonucleotides 5'-CTGGAGTCGACTTGGCGGACT-3' (SEQ ID NO: 19) and 5'-CGCGGATCCAAGCTTACTTACCTTCCATGCTGCCCTTATCCTCG-3' (SEQ ID NO: 20) and ligated as *SalI*-*Bam*HI fragment into the LTR-FLT4l vector replacing

the sequences encoding the transmembrane and cytoplasmic domains. This Flt4EC insert containing a splice donor site was ligated first into pHyCE2 containing exons encoding the human immunoglobulin heavy chain hinge and constant region exons (Karjalainen, K., *TIBTECH*, 9: 109-113 (1991)). The *EcoRI-BamHI* insert containing the Flt4-Ig chimera was then blunted by methods standard in the art (Klenow) and ligated to the blunted *HindIII* site in pREP7 (Invitrogen). The construct was transfected into 293-EBNA cells by the calcium-phosphate precipitation method and the conditioned medium was used for the isolation of the Flt4-Ig protein by protein A-Sepharose affinity chromatography.

#### EXAMPLES 15-17

##### Purification and sequencing the Flt4 ligand

Cell culture supernatants produced by PC-3 cells under serum-depleted conditions are concentrated 30-50 fold using Centriprep filter cartridges and loaded onto a column of immobilized Flt4 extracellular domain. Two affinity matrices are prepared using the alternative constructs and methods. In the first case the Flt4EC-6xHis fusion protein is crosslinked to CNBr-activated Sepharose 4B (Pharmacia) and in the second case the Flt4-Ig fusion protein is coupled to protein A Sepharose using dimethylpimelidate (Schneider *et al.*, 1982, *J. Biol. Chem.* 257: 10766-10769). The material eluted from the affinity column is subjected to further purification using ion exchange and reverse-phase high pressure chromatography and SDS-polyacrylamide gel electrophoresis. Chromatography fractions are tested for the ability to stimulate tyrosine phosphorylation of Flt4. The purified biologically active ligand protein is microsequenced and the degenerate oligonucleotides are made based on the amino acid sequence obtained, for the purpose of isolating and cloning a ligand-encoding cDNA; e.g., from a cDNA library generated from poly(A)<sup>+</sup> RNA isolated from PC-3 cells.

A detailed characterization of an Flt4 ligand, designated VEGF-C, is provided in PCT Patent Application No. PCT/US98/01973, filed 2 February 1998 (published as International Publication No. WO 98/33917); in PCT Patent Application PCT/FI96/00427, filed August 1, 1996 (published as International Publication WO

97/05250); and in the U.S. Patent Application priority documents relied upon therein for priority, all of which are incorporated herein by reference: U.S. Patent Application Serial Nos. 08/510,193, filed August 1, 1995; 08/585,895, filed January 12, 1996; 08/601,132, filed February 14, 1996; 08/671,573, filed June 28, 1996; and U.S. Patent Application Serial No. 08/795,430, filed February 5, 1997. It will be apparent from the foregoing teachings that the Flt4 ligand described herein may be used in assays as an additional indicia to confirm the identity of human *Flt4* allelic variants, and to confirm that non-human gene sequences having homology to the *Flt4* sequences taught herein (See, e.g., Example 8 and Fig. 4) are in fact the non-human counterparts to *Flt4*. The deduced amino acid sequence for prepro-VEGF-C is set forth herein in SEQ ID NO: 21.

A detailed description of a second Flt4 ligand, designated VEGF-D, is provided in Achen, *et al.*, *Proc. Nat'l Acad. Sci. U.S.A.*, 95(2): 548-553 (1998), also incorporated herein by reference. The cDNA and deduced amino acid sequences for prepro-VEGF-D are set forth herein in SEQ ID NO: 22.

## EXAMPLE 18

### Cloning of mouse *Flt4* cDNA probes

Approximately 10<sup>6</sup> plaques from a λFIX<sup>®</sup>II genomic library from 129SV mice (Stratagene) was screened with the S2.5 human Flt4 receptor cDNA fragment described above, covering the extracellular domain. See also Pajusola *et al.*, *Cancer Res.*, 52:5738 (1992). A 2.5 kb *Bam*HI fragment was subcloned from a positive plaque and sequenced from both ends. From this subclone, polymerase chain reaction was used to amplify and clone into the pBluescript KSII+/- vector (Stratagene) an exon fragment covering nucleotides 1745-2049 of the mouse *Flt4* cDNA sequence. See Finnerty *et al.*, *Oncogene*, 8:2293 (1993).

A second fragment covering nucleotides 1-192 was similarly cloned.

## EXAMPLE 19

### Analysis of *Flt4* mRNA in mouse tissues

Total RNA was isolated from developing embryos (8-18 days p.c. and one day old mice) according to Chomczynski et al., *Anal. Biochem.*, 162:156 (1987).

5 The sample from 8 day p.c. embryos also included the placenta.

For RNase protection analysis, RNA probe was generated from the linearized murine *Flt4* plasmid obtained according to Example 18 using [<sup>32</sup>P]-UTP and T7 polymerase for the antisense orientation. The  $\beta$ -actin probe used corresponds to nucleotides 1188-1279 of the published mouse  $\beta$ -actin sequence. See Tokunaga, *et*  
10 *al.*, *Nucleic. Acid. Res.*, 14:2829 (1986). After purification in a 6% polyacrylamide/7M urea gel, the labelled transcripts were hybridized to 30  $\mu$ g of total RNA overnight at 52 °C. Unhybridized RNA was digested with RNase A (10 U/ml) and T1 (1 mg/ml) at 37 °C, pH 7.5 for 1 hour. The RNases were inactivated by proteinase K digestion at 37 °C for 15 minutes and the samples were analysed in a 6%  
15 polyacrylamide/7M urea gel.

The pattern of expression of *Flt4* analysed in this experiment showed that very weak mRNA signals were obtained from lung, liver, heart, kidney, skeletal muscle and spleen, whereas testis and brain were apparently without specific signal. Analysis of a series of RNAs collected during different phases of mouse development  
20 by RNase protection assay showed that the *Flt4* mRNA was expressed throughout embryogenesis from day 8 p.c. to newborn mice without great variations in signal intensity.

## EXAMPLE 20

### *In situ* hybridization for *Flt4* in mouse embryos

25 To better assign *Flt4* transcripts to cells and tissues, sections of 7.5 and 8.5 day p.c. mouse embryos were hybridized with labelled *Flt4* RNAs. Mouse embryos were derived from matings of CBA and NMRI mice. Pregnant mice were killed by cervical dislocation and the embryos were either immediately frozen or transferred via phosphate buffered saline into 4% paraformaldehyde. The embryos and



isolated mouse organs were fixed for 18 hours at 4°C, dehydrated, embedded in paraffin, and cut into 6  $\mu$ m sections.

RNA probes (antisense and sense) of 192 and 305 nucleotides (see Example 18) were generated from linearized plasmids using [<sup>35</sup>S]-UTP. *In situ* hybridization of sections was performed according to Wilkinson *et al.*, *Development*, 99:493 (1987); and Wilkinson *et al.*, *Cell*, 50:79 (1987), incorporated by reference herein, with the following modifications: 1) instead of toluene, xylene was used before embedding in paraffin wax; 2) 6  $\mu$ m sections were cut, placed on a layer of diethyl pyrocarbonate-treated water on the surface of glass slides pretreated with 2% 3-triethoxysilylpropylamine; 3) alkaline hydrolysis of the probes was omitted; and 4) the high stringency wash was for 80 minutes at 65°C in a solution containing 30 mM DTT and 1 x SSC. The sections were covered with NTB-2 emulsion (Kodak) and stored at 4°C. The slides were exposed for 14 days, developed, and stained with hematoxylin. Control hybridizations with sense strand and RNase A-treated sections did not give a specific signal above background.

*Flt4* mRNA expression was not detected in 7.5 day p.c. mouse embryos, but bright signals were detected in the developing aortae on day 8.5 of development. In contrast, the developing yolk sac was *Flt4*-negative. In the extraembryonic tissues, *Flt4* was prominently expressed in the allantois, whereas developing blood islands of the yolk sac were negative. On the other hand, angioblasts of the head mesenchyme were strongly *Flt4*-positive. In the developing placenta, *Flt4* signal was first seen in peripheral sinusoidal veins. In 9.5 day p.c. placenta, the endothelium of venous lacunae and the giant cells partially fused to the Reichert's membrane expressed *Flt4* mRNA.

Thus, although *Flt4* expression was very prominent in the earliest endothelial cell precursors, the angioblasts, it appeared to be restricted only to certain vessels of 8.5 day p.c. embryos. The Tie receptor is known to be expressed in all endothelial cells of developing mouse embryos and thus provides a marker for these cells. See Korhonen, et al. *Oncogene*, 8:395 (1993); and Korhonen et al., *Blood*, 80: 2548-2555 (1992). Notably, in contrast to the Tie probe, the *Flt4* probe hybridized

very weakly if at all with arterial endothelia of 11.5 day p.c. embryos, e.g. with the endothelium of the developing dorsal aorta or the carotid arteries. Instead, Flt4 signal was much more prominent in the developing veins. For example, Flt4 signal was detected in veins surrounding the developing metanephros, while the Tie probe  
5 predominantly recognized capillaries within the metanephros.

*Flt4* mRNA was observed to be distributed in several regions of a 12.5 day p.c. mouse embryo, being particularly prominent in the dilated vessel of the axillar region. A similar Flt4-positive vessel structure was seen in the mid-sagittal section in the jugular area (data not shown). A plexus-like pattern of *Flt4*-expressing vessels  
10 appeared in the periorbital region and surrounding the developing vertebrae. Also, just beneath the developing skin, a Flt4-positive vascular network was evident. Weaker capillary signals were obtained from several regions, including the developing brain. *Flt4* mRNA could also be detected in small vessels of the neck region, of the developing snout and at the base of the developing tongue as well as in the tail region.  
15 Additionally, the liver was strongly positive for *Flt4* mRNA in a spotlike pattern.

During further development, *Flt4* mRNA appeared to become more restricted to certain vessels of the embryo. A 14.5 day p.c. embryo shows nicely this restricted pattern of expression. In the midsagittal section from such an embryo, the most prominent *Flt4* signal was observed along the developing vertebral column in its  
20 anterior part. This signal was considered to originate from endothelial cells of the thoracic duct, which is the largest lymphatic vessel formed at this time of development. In contrast, the dorsal aorta and inferior vena cava were negative. Dilated vessels in the mesenteric region were also strongly positive for Flt4. Furthermore, as in the 12.5 day p.c. embryos, vessel networks along anatomical boundaries in the periorbital,  
25 lower jaw, as well as in the neck regions contained Flt4-positive endothelia. Similar structures were present in the pericardial space and throughout the subcutaneous tissue. Notably, in contrast to Flt4-negative vessels, all Flt4-positive vessels were devoid of blood cells in their lumen. These expression patterns suggest that Flt4 becomes confined to the endothelia of lymphatic vessels at this time of development. An

additional site where we observed *Flt4* expression was in the sinusoids of the developing bone marrow.

A transverse section of the upper thorax of a 16.5 day p.c. embryo hybridized with the *Flt4* probe also was analyzed. Hematoxylin-eosin staining was performed to visualize the different types of vessels in this area. These include the carotid and brachiocephalic arteries, the vena cava, and the thoracic duct, which is smaller in size and lacks surrounding muscular and connective tissue. Under higher magnification endothelial cells of the thoracic duct as well as a small vessel in the vicinity were observed to hybridize with the *Flt4* probe.

## EXAMPLE 21

### Analysis of *Flt4* mRNA in cultured endothelial cells

The *in situ* hybridization results described in Example 20 showed that *Flt4* is expressed in venous endothelial cells and later in lymphatic vessels and some venous endothelial cells, but not in arterial endothelia. In order to determine if such regulation was maintained *in vitro*, we studied cultured endothelial cells using Northern blotting and hybridization analysis.

Endothelial cells from human aorta, femoral vein, umbilical vein, and from foreskin microvessels were isolated, cultured, and characterized as previously described in the art. See Van Hinsberg et al., *Arteriosclerosis*, 7:389 (1987); and Van Hinsberg, et al., *Thromb. Haemostas*, 57:148 (1987). They were used at confluent density after five to eight passages (split ratio 1:3) for the isolation of polyadenylated RNA.

The endothelial cell lines EA.hy926 (Edgell et al., *Proc. Natl. Acad. Sci.*, 80: 3734-3737 (1983)), BCE (Folkman et al., *Proc. Natl. Acad. Sci.*, 76: 5217-5221 (1979)) and LEII (Schreiber et al., *Proc. Natl. Acad. Sci.*, 82: 6138 (1985)) did not express *Flt4*. However, cultured human microvascular, venous and umbilical vein endothelial cells were positive for the *Flt4*-specific 5.8 and 4.5 kb mRNAs, whereas the aortic endothelial cells were negative. In contrast, another endothelial receptor

tyrosine kinase gene, *tie*, was expressed as a 4.4 kb mRNA in all endothelial cell types studied.

## EXAMPLE 22

### *Flt4* mRNA in in adult human tissues

5                   The results obtained in Example 20 indicated that the *Flt4* mRNA becomes largely confined to the endothelium of lymphatic vessels during development. Because of the potential significance of this finding in humans, we also studied *Flt4* expression in adult human tissues using a human *Flt4* probe. The human *Flt4* probe used was an *EcoRI-SphI* fragment covering base pairs 1-595 of the cDNA (SEQ ID  
10 NO:1). See also Pajusola et al., *Cancer Res.*, 52:5738 (1992). The von Willebrand factor probe was an *EcoRI-HindIII* fragment covering base pairs 1-2334. Bonthron, et al., *Nucleic Acids Res.*, 141:7125 (1986).

                  We used routinely fixed material sent for histopathological diagnosis. Normal lung tissue was obtained from a resection of the left inferior lung lobe affected  
15 by epidermoid cancer. Mesenterium and mesenterial lymph nodes were obtained from a patient having a colonic adenocarcinoma. A normal lymph node adjacent to the salivary gland was enucleated because of its abnormal size. The tonsils from two patients and the two appendixes had no diagnostic changes. Two lymphangiomyomas and three cystic lymphangiomas were studied with similar results.

20                   For human tissues, which were routine samples fixed with 10% formalin for histopathological diagnosis, the normal *in situ* protocol gave just background, whereas microwave treatment instead of proteinaase K enabled specific hybridization. Shi, et al., *J. Biol. Chem.*, 266:5774 (1991); Catoretti, et al., *J. of Pathol.*, 168:357 (1992).

25                   In the mesenterium, lung and appendix lymphatic endothelia gave *Flt4* signals, while veins, arteries, and capillaries were negative. To study whether *Flt4* is expressed in the HEVs, the tonsils were studied. Indeed, in the tonsils, *Flt4*-specific autoradiographic grains were detected in some HEVs.

### EXAMPLE 23

#### **Analysis of *Flt4* mRNA in normal and metastatic lymph node and in lymphangioma**

A portion of a human mesenteric lymph node (see Example 22) was analysed for *Flt4* expression. *Flt4* expression was observed in the lymphatic sinuses and afferent and efferent lymphatic vessels. The same pattern was observed in a lymph node containing adenocarcinoma metastases. Some HEVs in both normal and metastatic lymph node were also positive. *Flt4* expression in a cystic lymphangioma was specific to lymphatic endothelia, as evident from a comparison with the *in situ* signals for von Willebrandt factor in all blood vessels.

Consistent with these results, immunostaining for Flt4 was strongly positive in the endothelium of cutaneous lymphangiomatosis, a rare disorder characterized by proliferation of presumed lymphatic endothelium. See Lymboussaki *et al.*, *Am. J. Pathol.*, 153(2): 395-403 (August, 1998), incorporated herein by reference in its entirety.

Additionally, immunostaining for Flt4 identified spindle cells within Kaposi's sarcoma cutaneous nodular lesion tissue samples. See Jussila *et al.*, *Cancer Res.*, 58:1599-1604 (April, 1998). In view of the apparent lymphatic specificity of Flt4, These results may be considered consistent with suggestions that certain cells in Kaposi's sarcoma are of lymphatic endothelial origin. See, e.g., Beckstead *et al.*, *Am J. Pathol.*, 119: 294-300 (1985); and Dictor *et al.*, *Am J. Pathol.*, 130: 411-417 (1988).

### EXAMPLE 24

#### **Localization of Flt4 in fetal endothelial cells**

As described in Example 2, An *Flt4* cDNA fragment encoding the 40 carboxy terminal amino acids of the short form was cloned as a 657 bp *EcoRI*-fragment into the pGEX-1λT bacterial expression vector (Pharmacia) in frame with the glutathione-S-transferase coding region. The resultant GST-Flt4 fusion protein was produced in *E.coli* and purified by affinity chromatography using a glutathione-Sepharose 4B column. The purified protein was lyophilized, dissolved in PBS, mixed

with Freund's adjuvant, and used for immunization of rabbits. Antisera were used after the third booster immunization.

5 Tissues from 17 and 20-week-old human fetuses were obtained from legal abortions induced with prostaglandins. The study was approved by the Ethical Committee of the Helsinki University Central Hospital. The gestational age was estimated from the fetal foot length. The fetal tissues were embedded in Tissue-Tek (Miles), frozen immediately, and stored at -70 °C.

Anti-Flt4 antiserum was cross-absorbed to a GST-Sepharose column to remove anti-GST-antibodies and then purified by GST-Flt4 affinity  
10 chromatography. Several 6 µm-thick cryostat sections of the tissues were fixed with acetone and treated with 0.3% H<sub>2</sub>O<sub>2</sub> in methanol for 30 minutes to block endogenous peroxidase activity. After washing, the sections were incubated with 5% normal swine serum. Sections were then incubated with antibodies against Flt4 and washed. Bound antibodies were detected with peroxidase-conjugated swine anti-rabbit IgG followed  
15 by staining for peroxidase activity using 0.2% 3,3-diaminobenzidine (Amersham) as a substrate. The sections were counterstained in Meyer's hematoxylin.

Anti-Flt4 immunoperoxidase staining of human fetal mesenterium showed Flt4 protein in the endothelium of several vessels, while control stainings with antigen-blocked anti-Flt4 antibodies and preimmune sera were negative. For  
20 comparison, sections were stained with an antiserum against the Factor VIII-related antigen, which is specific for vascular endothelial cells. Immunoperoxidase staining for Flt4 was observed over endothelial cells of vessels, which did not contain red blood cells, while blood vessels were negative. The vessels without red blood cells are likely to be lymphatic endothelial cells; such vessels are particularly frequent in the  
25 mesenterium. The antibodies against Factor VIII related antigen stained endothelial cells in all vessels.

## EXAMPLE 25

### Production of monoclonal antibodies against Flt4

#### Fusion I:

Recombinant Flt4 extracellular domain protein was produced by  
5 expressing the Flt4EC-6xHis-pVTBac plasmid construct (Example 14) in High-Five  
cells. The Flt4 extracellular domain (Flt4EC) was purified from the culture medium of  
the infected High-Five cells using Ni-NTA affinity chromatography according to  
manufacturer's instructions (Qiagen) for binding and elution of the 6xHis tag encoded  
in the COOH-terminus of the recombinant Flt4 extracellular domain.

10 Four month old Balb/c male mice were immunized by intraperitoneal  
injection of the purified, recombinantly produced Flt4 extracellular domain protein  
(150  $\mu$ g/mouse) emulsified with Freund's complete adjuvant. Booster injections of 150  
 $\mu$ g were given at three to four week intervals and a final booster (10  $\mu$ g Flt4 EC in  
PBS, administered intraperitoneally) was given after another three-week interval. Four  
15 days after the final booster dose, the mice were sacrificed and mouse splenic lymphoid  
cells were fused with SP 2/0 plasmacytoma cells at a 2:1 ratio, respectively.

The fused cells were harvested in 96-well culture plates (NUNC) in  
Ex-Cell 320 medium (SERALAB) containing 20% fetal calf serum and HAT  
supplement (hypoxanthine-aminopterin-thymidine; GIBCO, 043-01060H; diluted 50-  
20 fold). Cells were cultured at +37°C, in a 5% CO<sub>2</sub> atmosphere. After 10 days, HAT-  
supplemented medium was changed to HT-supplemented cell culture medium  
(GIBCO; 043-01065H, diluted 50-fold). HT medium is identical to HAT medium, but  
lacks aminopterin.

In three weeks, specific antibody production was determined by the  
25 antigen-specific ImmunoFluoroMetric Assay, (IFMA), described below in Example 26.  
The master clones were cloned by limited dilutions as described by Staszewski *et al.*,  
*Yale Journal of Biology and Medicine*, 57:865-868 (1984). Positive clones were  
expanded onto 24-well tissue culture plates (NUNC), recloned, and re-tested by the  
same method. Positive clones were tested by fluorescence-activated cell sorting  
30 (FACS).

The stable clones secreted immunoglobulins belonging to the IgG<sub>1</sub> class, except one, which produced Ig probably belonging to class IgA. The subclass of monoclonal antibody was determined using rat monoclonal antibody to mouse subclass as biotin conjugate (SEROTEC) in IFMA.

5                    Balb/c mice were used to produce monoclonal antibodies in ascites fluid. The hybridomas described above were intraperitoneally injected into mice after pretreatment of the animals with pristane (2,6,10,14-tetramethylpentadecan 98%, ALDRICH-CHEMIE D7924 Steinheim, Cat.No. T 2,280-2). 0.5 ml of pristane (i.v.) was injected about two weeks prior to the hybridoma cells. The amount of cells  
10 injected were approximately  $7.5$  to  $9 \times 10^6$  per mouse. Ascites was collected 10 to 14 days after injection of the hybridomas.

#### Fusion II:

Two month old Balb/c mice (female) were immunized by intraperitoneal injection of the recombinantly produced Flt4 extracellular domain protein (20  
15  $\mu\text{g}/\text{mouse}$ ), emulsified with Freund's complete adjuvant. Booster injections of 20  $\mu\text{g}$  were given at three to four week intervals and a final booster (10  $\mu\text{g}$  Flt4 in PBS, administered i.v.) was given after another three-week interval. Four days after the final booster dose, the mice were sacrificed and mouse splenic lymphoid cells were fused with SP 2/0 plasmacytoma cells at a 2:1 ratio, respectively.

20                    The fused cells were harvested in 96-well culture plates (FALCON) in OptiMEM 1 (with Glutamax, 1, 51985-026, GIBCO BRL) medium containing 20% fetal calf serum and HAT supplement (hypoxanthine-aminopterin-thymidine, GIBCO BRL 21060-017; diluted 1:50 fold). Cells were cultured at 37°C, in a 5% CO<sub>2</sub> atmosphere. After 10 days, HAT-supplemented medium was changed to HT-  
25 supplemental cell culture medium (GIBCO BRL; 41065-012, diluted 1:50-fold).

In three weeks, specific antibody production was determined by the antigen-specific ImmunoFluoroMetric Assay (IFMA) described below in Example 26. The master clones were cloned by limited dilutions as described by Staszewski et al.



(1984). Positive clones were expanded onto 24-well tissue culture plates (FALCON), re-cloned, and re-tested by the same method. Positive clones were tested by FACS.

The 2E11 and 6B2 clones secreted immunoglobulins belonging to the IgG<sub>1</sub> class, and 2B12 clones produced Ig belonging to subclass IgM. The mouse  
5 subclass IgG<sub>1</sub> was determined using rat monoclonal antibody against mouse subclass heavy chain as biotin conjugate (SEROTEC) in IFMA and the mouse subclass IgM was determined with Mouse Monoclonal Antibody Isotyping Kit (Dipstick Format) (19663-012, Life Technologies Inc.).

## EXAMPLE 26

### 10 Specificity of monoclonal antibodies against Flt4

The purified, recombinant Flt4 extracellular domain-6xHis fusion product (produced as described in Examples 14 and 25) was labelled with Europium according to Mukkala et al., *Anal.Biochem*, 176(2):319-325 (1989), with the  
15 following modification: a 250 times molar excess of isothiocyanate DTTA-Eu (N1 chelate, WALLAC, Finland) was added to the Flt4 solution (0.5 mg/ml in PBS) and the pH was adjusted to about 9 by adding 0.5 M sodium carbonate buffer, pH 9.8. The labelling was performed overnight at +4°C. Unbound label was removed using PD-10 (PHARMACIA, Sweden) with TSA buffer (50 mM Tris-HCl, pH 7.8, containing 0.15 M NaCl) as eluent.

20 After purification, 1 mg/ml bovine serum albumin (BSA) was added to the labelled Flt4 and the label was stored at +4°C. The average number of Europium ions incorporated per Flt4 molecule was 1.9, as determined by measuring the fluorescence in a ratio to that of known EuCl<sub>3</sub> standards (Hemmilä et al., *Anal.Biochem.*, 137:335-343 (1984)).

25 The antibodies produced in Example 25 were screened using a Sandwich-type immunofluorometric assay, using microtitration strip wells (NUNC, polysorb) coated with rabbit anti-mouse Ig (Z 259, DAKOPATTS). The pre-coated wells were washed once by Platewash 1296-024 (WALLAC) with DELFIA wash solution. The DELFIA assay buffer was used as a dilution buffer for cell culture

supernatants and for serum of the splenectomized mouse (at dilutions between 1:1000 to 1:100,000) used as positive control in the preliminary screening assay.

An overnight incubation at +4°C (or alternatively for 2 hours at room temperature) was begun by shaking on a Plateshake shaker (1296-001, WALLAC) for 5 minutes followed by washing four times with wash solution as described above.

The Europium-labelled Flt4 was added at a dilution of 1:500 in 100 µl of the assay buffer. After 5 minutes on a Plateshake shaker and one hour incubation at room temperature, the strips were washed as described above.

Enhancement solution (DELFLIA) was added at 200 µl/well. The plates were then shaken for 5 minutes on a Plateshake shaker and the intensity of fluorescence was measured by ARCUS-1230 (WALLAC) for 10-15 minutes. (Lövgren et al., In: Collins W.P. (Ed.) Alternative Immunoassays, John Wiley & Sons Ltd. (1985), pp. 203-216). The DELFLIA results show that all monoclonal antibodies tested bound the Flt4 EC antigen. Monoclonal antibodies reactive with the Flt4 (and the hybridomas which produce the antibodies) were selected for further screening.

The resulting monoclonal antibodies were used in double antibody immunofluorescence staining of NIH3T3 cells expressing the LTR-FLT4l construct and neomycin-resistant transfected NIH3T3 cells. The cells were detached from the culture plates using EDTA, stained, and analysed in a fluorescence-activated cell sorter (FACS). The results of FACS analysis are given as percentages of cells staining positive with the indicated monoclonal antibody (see Table 2, below).

TABLE 2			
Mab clones	LTR% <sup>a)</sup>	NEO% <sup>b)</sup>	DELFLIA-counts
1B1	67.3	1	20625
1B1D11	75	1.2	19694
1B1F8	76.1	1.4	18580
4F6	69.9	1.2	23229
4F6B8G12	75	0.3	24374

TABLE 2			
Mab clones	LTR% <sup>a)</sup>	NEO% <sup>b)</sup>	DELFLA-counts
4F6B8H11	75.9	0.3	28281
4F6B8E12	74.8	0.4	27097
4F6B8G10	75.3	0.4	26063
9D9	45.1	0.75	17316
9D9D10	71.7	2.3	18230
9D9F9	73	1.8	11904
9D9G6	74.3	2.9	16743
9D9G7	70.7	1.3	17009
10E4	24.2	1.4	39202
10E4B10E12	32.3	0.3	42490
10E4B10G10	36.5	0.3	54815
10E4B10F12	45.6	0.4	43909
10E4B10G12	45.7	0.5	35576
11G2	30.2	1.6	11304
11G2D12	74.4	1.5	14660
11G2G9	74.2	0.9	10283
11G2H7	74.4	2.1	25382

a) FACS results with LTR transfected cells

b) FACS results with NEO cells (control)

The FACS results with LTR-FLT4l-transfected cells indicate that the antibodies effectively recognize Flt4-expressing cells. These same antibodies give only background staining of neomycin phosphotransferase-trasfected NIH3T3 cells. Thus, the antibodies specifically recognize the Flt4 tyrosine kinase on the cell surface.

One clone, designated anti-Flt4 hybridoma 9D9F9, was found to stably secrete monoclonal antibody which was determined to be of immunoglobulin class

IgG<sub>1</sub> by IFMA. Hybridoma 9D9F9 was deposited with the German Collection of Microorganisms and Cell Cultures, Department of Human and Animal Cell Cultures and Viruses, Mascheroder Weg 1b, 3300 Braunschweig, Germany, March 23, 1995, and given accession No. ACC2210.

5     Fusion II antibodies

           The Europium-labelled Flt4 extracellular domain protein described above also was used to screen the Fusion II antibodies described in Example 25. The antibodies were screened using a Flt4-specific IFMA using microtitration wells (Nunc, Polysorb) coated with rabbit anti-mouse Ig (Z 259, DAKO). The precoated wells  
10     were washed once with wash solution (Wallac) by using DELFIA Plate wash.

           The DELFIA assay buffer was used as dilution buffer for cell culture supernatants (dilution 1:2 in preliminary screening) and for serum of the splenectomized mouse (dilutions 1:1000 to 1:100,000) which was used as a positive control. As standard, the purified anti-Flt4 9D9F9 (mouse subclass IgG<sub>1</sub>) was used at  
15     concentrations between 1.0 ng/ml and 250 ng/ml. Samples were first shaken at room temperature for five minutes on a Plateshake shaker and then incubated approximately 18 hours at +4°C. The frames were first washed four times, then the Eu-labelled Flt4 (1:2000, in 100 µl assay buffer) was added, and finally the frames were incubated for one hour at room temperature. After washing as described, the enhancement solution  
20     (200 µl/well, Wallac) was added, and the frames were shaken for 5 minutes on the Plateshake shaker. The intensity of fluorescence was measured by ARCUS-1230 (Wallac). Monoclonal antibodies reactive with Flt4 were selected for further screening in the double antibody immunofluorescence staining assay employing Flt4-expressing  
25     NIH3T3 cells, as described above.

           The resulting Fusion II monoclonal-antibodies against Flt4 and corresponding results of FACS analysis (expressed as percentages of cells staining positive with the indicated monoclonal antibody) are summarized in Table 3.

A standard curve for quantitation of anti-Flt4 antibodies was made by using affinity purified anti-Flt4 9D9F9. The linear range reached from 1.0 ng/ml to 250 ng/ml.

Cell lysate of NIH3T3 cells co-transfected with pLTRFLT4l construct expressing full-length Flt4 on the surface was electrophoresed in 6.5% SDS-PAGE, proteins were transferred onto nitrocellulose nitrate membrane (0.45  $\mu$ m, SCHLEICHER & SCHUELL) and immunoblotted with monoclonal antibody-containing hybridoma cell culture supernatants (1:10, 50 mM TRIS - 40 mM glycine buffer containing methanol 4%, SDS 0.04%). The specificities of monoclonal antibodies were detected using incubation with HRP-conjugated rabbit antimouse Ig (P 161, DAKO, diluted 1:1000 in 20 mM TRIS buffer, pH 7.5, containing 150 mM saline, 5% milk powder) and ECL (Enhanced chemiluminescence, AMERSHAM).

TABLE 3

Mab clones	LTR % <sup>a)</sup>	NEO <sup>b)</sup>	approx. Mab production ng/ml/10 <sup>6</sup> cells <sup>c)</sup>	WB
2B12E10	39.5	6.0	440	+
2E11D11	44.6	8.8	110	+
2E11F9	49.5	4.5	100	+
2E11F12	46.0	4.1	180	+
2E11G8	41.2	7.8	160	+
6B2E12	NF	NF	1390	+
6B2F8	NF	NF	470	+
6B2G6	NF	NF	630	+
6B2H5	NF	NF	740	+
6B2H8	NF	NF	1800	+

a) FACS results with LTR transfected cells

- b) FACS results with NEO cells (control)
- c) quantitation of Mab production based on affinity-purified antiFLT 9D9F9 antibody used as standard

5 NF not functioning in FACS

WB Used successfully in Western immunoblotting

### EXAMPLE 27

#### **Use of anti-Flt4 antibodies to identify Flt4 in cell lysates and expressed in lymphatic endothelial cells in human tissue**

10

The monoclonal antibodies produced by hybridoma 9D9 described in the preceding examples were used in immunoprecipitation and Western blotting of lysates of HEL cells. As reported in Example 6, *Flt4* mRNA expression had been previously observed in HEL cells. About  $2 \times 10^7$  cultured HEL cells were lysed in  
15 RIPA buffer specified in Example 11 and immunoprecipitated with about 2 micrograms of the 9D9 antibody (as described for polyclonal antibodies in example 11). For Western analysis, immunoprecipitates were electrophoresed via SDS-PAGE (6% gel) and electroblotted onto a nitrocellulose membrane. Polypeptide bands of 175 kD and 125 kD, corresponding to Flt4 polypeptides, were detected in the Western blotting  
20 analysis of the immunoprecipitates using a 1 microgram/ml dilution of the 9D9 antibody.

Immunostaining of human skin tissue was performed using the 9D9 monoclonal antibodies and an alkaline phosphatase ABC-AP kit (Dako). Briefly, slides containing 6  $\mu$ m samples of adult human skin were dried for 30 minutes at room  
25 temperature (RT), fixed for ten minutes with cold acetone, and then washed once for five minutes with phosphate-buffered saline (PBS). The samples were then incubated for 30 minutes at RT with 2% horse serum and washed three times for five minutes in PBS.

For immunostaining, the samples were incubated for one hour at RT  
30 with the 9D9 primary antibody and washed three times for five minutes with PBS. After washing, the samples were incubated for thirty minutes at RT with biotinylated

rabbit anti-mouse secondary antibodies, and again washed three times for five minutes with PBS.

Bound antibodies were detected by incubating the samples for thirty minutes at RT with ABC-AP complex, washing three times with PBS, incubating for fifteen minutes at RT with AP-substrate (Sigma Fast Red TR/Naphtol AS-MX (Cat. No. F-4648)), and rinsing with water. Samples were then counter-stained with Mayer's hematoxylin for thirty seconds and rinsed with water. Aquamount and a coverslip were applied, and the samples were analyzed under a microscope. The 9D9 antibody staining was observed in lymphatic endothelial cells in these human skin sections. Blood vessel endothelia showed extremely weak or no staining. Additional analyses have served to confirm the apparent specificity for lymphatic endothelia. See Lymboussaki *et al.*, *Am. J. Pathol.*, 153(2):395-403 (August, 1998); and Jussila *et al.*, *Cancer Res.*, 58:1599-1604 (April, 1998), both of which are incorporated herein by reference in their entireties.

These results further confirm the utility of Flt4 as a useful marker for lymphatic endothelia and the utility of anti-Flt4 antibodies for identifying and visualizing Flt4 expressed in these cells, in a tissue sample.

## EXAMPLE 28

### **Upregulation of the VEGF-C/VEGFR-3 signalling pathway in breast cancer angiogenesis**

The foregoing examples demonstrate that Flt4 (VEGFR-3) is useful as a specific antigenic marker for lymphatic endothelia in normal tissues. The following procedures additionally demonstrate that VEGFR-3 is useful as an antigenic marker (e.g., for diagnosis and screening) and as a therapeutic target in malignant breast tumors. A highly elevated number of VEGFR-3 positive vessels was found in invasive breast cancer in comparison to histologically normal breast tissue ( $P < 0.0001$ ).

## MATERIALS AND METHODS

Freshly frozen breast tissue samples were retrieved from the files of the Department of Pathology, University of Helsinki. The samples consisted of ductal carcinoma (n=6), lobular carcinoma (n=6), intraductal carcinoma (n=8), fibroadenoma (n=4), and histologically normal breast tissue (n=12). All samples had been frozen immediately after surgical excision in liquid nitrogen, and stored at -70°C.

Mouse monoclonal antibodies (Mabs) against human Flt4 (VEGFR-3) were produced essentially as described in preceding examples, e.g., Example 25. The VEGFR-3 extracellular protein domain (VEGFR-3EC) was expressed via a recombinant baculovirus in insect cells, purified from the culture medium. Mouse monoclonal antibodies against VEGFR-3EC were then produced using standard methods and the immunoglobulin fraction was purified by protein A affinity chromatography from hybridoma ascites fluid or Tecnomouse® culture supernatants.

Five  $\mu\text{m}$  cryosections of the tissues samples were air-dried and fixed in cold acetone for 10 minutes. The sections were re-hydrated in phosphate buffered saline (PBS) and incubated for 30 minutes in 5 % normal horse serum at room temperature. The sections were then incubated for 2 hours in a humid atmosphere at room temperature with the Mabs 9D9F9 (Example 26) at the concentration of 1.0  $\mu\text{g}/\text{ml}$ . Other anti-VEGFR-3 Mab against distinct epitopes of the VEGFR-3EC were also studied; clones 2E11D11 (Example 26) and 7B8F9 (made essentially as described in Example 26) were used at the concentrations of 9.5 and 8.5  $\mu\text{g}/\text{ml}$ , respectively. A subsequent incubation for 30 minutes in biotinylated anti-mouse serum was followed by a 60 minute incubation using reagents of the Vectastain Elite Mouse IgG ABC kit (Vector laboratories, Burlingame, USA). Peroxidase activity was developed with 3-amino-9-ethyl carbazole (AEC, Sigma, St. Louis, USA) for 10 minutes. Finally, the sections were stained with haematoxylin for 20 seconds. Negative controls were performed by omitting the primary antibody, or by using irrelevant primary antibodies of the same isotype. The purified baculoviral immunogen was used to block the binding of the 9D9 antibodies as another negative control. In these experiments, the antibodies were incubated overnight with a 10-fold molar excess of the VEGFR-3EC



protein in PBS. After centrifugation for 4 minutes at 4000 rpm, +4 °C, the supernatant was carefully collected and then used as primary antibody. The 5 µm cryosections adjacent to the ones stained with the anti-VEGFR-3 antibodies were immunostained for the blood vascular endothelial marker PAL-E (0.15 µg/ml, Monosan, Uden, the Netherlands ), laminin (1:4000 dilution of the supernatant of clone LAM-89, Sigma, St Louis, MO), collagen XVIII (1.9 µg/ml), α-smooth muscle actin (SMA, 0.5 µg/ml, clone 1A4, Sigma), VEGFR-1 (1:200 dilution of the supernatant of clone 19) or VEGFR-2 (dilution 1:100).

Pathological examination of all of the samples was performed after the staining procedures. The blood vascular densities were obtained from the slides stained for PAL-E [de Waal *et al.*, *Am. J. Pathol.*, 150: 1951-1957 (1997)], following the guidelines recommended by Gasparini and Harris. [Gasparini G, and Harris A, *J. Clin. Oncol.*, 13: 765-782 (1995).] The VEGFR-3 positive vessel densities were studied in the same way. A slide was first scanned at low magnification, and intratumoral vessel density was then assessed by counting the number of stained vessels per a 400x magnification high power field (hpf) in the areas with the highest vascular density ("vascular hotspots") or in the areas with highest VEGFR-3 positive vessel density. A minimum of 5 fields was counted per a slide, after which the 3 highest counts were averaged.

Double staining was performed to differentiate immunohistochemical staining of lymphatic and blood vessels in two intraductal carcinomas. Acetone-fixed 5 µm cryosections were incubated for 1 hour with anti-PAL-E antibodies, with biotinylated horse anti-mouse antibody (Vectastain Elite Mouse IgG ABC kit, Vector laboratories, Burlingame, USA) for 30 minutes, with ABC-peroxidase (Vectastain, 1:100) for 45 minutes, and developed finally with AEC for 10 minutes. For the second step, the sections were incubated with anti-VEGFR-3 antibodies for 1 hour (0.14 µg/ml), followed by biotinylated anti-mouse antibody for 30 minutes (1:200 dilution of the supernatant of clone), ABC-peroxidase for 30 minutes (1:100), biotinylated tyramin solution (1:2.000) containing 0.01% peroxide for 5 minutes, ABC-alkaline phosphatase (1:100) for 20 minutes, and developed with Fast Blue (Sigma, St. Louis,

USA) for 20 minutes, according to a procedure previously described in the literature for ISH signal enhancement. [Kerstens *et al.*, *J. Histochem. Cytochem.*, 43: 347-352 (1995).] Cryosections (5  $\mu$ m) adjacent to the double-stained sections were also immunostained with VEGFR-3 antibodies only, as described above.

5 Polyclonal antibodies were produced in rabbits against a synthetic peptide corresponding to the amino acid residues 2-18 of the N-terminus of mature, secreted human vascular endothelial growth factor C (VEGF-C) (residues 104-120 of the VEGF-C prepro-VEGF-C polypeptide) as described in the literature. [Joukov *et al.*, *EMBO J.*, 16: 3898-3911 (1997), incorporated herein by reference in its entirety.]  
10 The antisera were affinity-purified using the immunogenic polypeptide coupled to an epoxy-activated sepharose-6B column and tested for specific staining of VEGF-C using cells infected with an adenoviral vector expressing VEGF-C or control  $\beta$ -galactosidase.

The eight intraductal carcinomas and all of the invasive carcinomas  
15 analysed for VEGFR-3 were chosen for further analyses of the expression of VEGF-C. Five micrometer cryosections adjacent to the sections stained with the anti-VEGFR-3 antibodies were air-dried and fixed in cold acetone for 10 minutes. The sections were rehydrated in PBS and incubated for 30 minutes in 5% normal goat serum and then for 2 hours in a humid atmosphere at room temperature with the rabbit polyclonal  
20 antibodies against human VEGF-C, diluted 1:200 in PBS. A subsequent incubation for 30 minutes in biotinylated anti-rabbit serum was followed by a 60 minutes incubation using reagents of the Vectastain Elite Rabbit IgG ABC kit (Vector laboratories, Burlingame, USA). The sections were further processed as described above. As a negative control, the purified immunogen was used to block the binding of the VEGF-  
25 C antibodies. In these experiments, VEGF-C antibodies were incubated overnight with a 10-fold molar excess of the VEGF-C protein in PBS. After centrifugation for 4 minutes at 4000 rpm at +4 °C, the supernatant was carefully collected and used in the immunostainings.

Monoclonal antibodies to human type XVIII collagen were generated  
30 by DiaBor Ltd. (Oulu, Finland) by immunization of mice with the recombinant

polypeptide QH48.18 [Saarela *et al.*, *Matrix Biology*, 16: 319-28 (1998)], corresponding to the common region of the N-terminal NC1 domain of human type XVIII collagen. The clones were screened by ELISA assay and Western analysis using the polypeptide QH48.18, and also by immunofluorescence staining of frozen human tissue sections. The screening of the hybridoma clones resulted in three monoclonal antibodies, which were positive in all three assays mentioned (ELISA, Western, immunofluorescence staining). One of the antibodies which gave the strongest signals, DB144-N2, was used in subsequent experiments. It gave an identical staining pattern (e.g., in adult human skin and kidney samples) to that of the polyclonal anti-all hu(XVIII).

## RESULTS

### A. VEGFR-3 in histologically normal breast tissue and in benign fibroadenomas

Immunohistochemical staining of VEGFR-3 in normal breast tissue showed a very weak staining in capillaries of the interductal stroma. These vessels did not form any specific pattern, but were scattered throughout the stroma. The density of the VEGFR-3 positive vessels in the normal breast tissue samples ranged from 6 to 17 per hpf, median 9 (n=12). Most of such vessels were strongly stained for the blood vascular endothelial marker PAL-E and for the basal lamina component, collagen XVIII, suggesting that VEGFR-3 was expressed weakly in the blood vessels of normal breast tissue. However, some thin vessels in the stroma, which were clearly stained for VEGFR-3 were negative for PAL-E and only weakly positive for the collagen type XVIII, suggesting that they were lymphatic vessels. VEGFR-3 positive vessels were also uniformly found in benign fibroadenomas, where their density (median 8 vessels per hpf, range 3-19; n= 4) did not differ from that of the histologically normal breast tissue (median 8 vs. 9; P>0.1, the Mann-Whitney test).

### B. VEGFR-3 positive vessels in intraductal carcinomas

In intraductal carcinomas, a distinctive pattern of strongly-stained VEGFR-3 positive vessels was observed. The vessels formed arch-like structures

around the affected ducts (Fig. 5A). This "necklace" pattern also was observed in staining of adjacent sections for the blood vessel endothelial marker, PAL-E (Fig. 5B), suggesting that VEGFR-3 expression was enhanced in capillary endothelium. In order to more definitively differentiate between blood and lymphatic vessels and to search for the presence of smooth muscle cells and pericytes in the vessel walls, additional stainings were done using antibodies against smooth muscle  $\alpha$ -actin (SMA) and basal lamina components laminin and type XVIII collagen. According to this staining, the small vessels close to the intraductal carcinomas expressed simultaneously VEGFR-3 and the basal lamina proteins, but stained more weakly for SMA, indicating that they are incompletely covered by pericytes/smooth muscle cells in the vessel wall (black arrows in Figs. 5C-5F). In contrast, larger blood vessels at some distance from the intraductal lesions were in general negative for VEGFR-3, but positive for laminin, collagen XVIII and SMA (red arrows). In addition, vessels were found, which were positive for VEGFR-3, but only very weakly stained for the basal lamina proteins laminin and type XVIII collagen and not at all for SMA (green arrows). These were considered to represent lymphatic vessels.

C. Differential double-staining of blood and lymphatic vessels

Two intraductal carcinomas were chosen for the immunohistochemical double-staining procedure to more clearly differentiate lymphatic vessels from blood vessels. [See de Waal *et al.*, *Am. J. Pathol.*, 150: 1951-1957 (1997).] Using this method, the VEGFR-3 positive vessels were stained blue, while the PAL-E positive vessels and basal laminae were stained brown. Both tested samples showed a similar pattern of staining: the vessels lining the tumor filled ducts were predominantly PAL-E positive (arrowhead in Figures 5G and 5H) while the presumably lymphatic, VEGFR-3 positive vessels a short distance away in the interductal stroma were PAL-E negative (black arrows in Figs 5G and 5H). In order to exclude misinterpretation due to possible double-staining artefacts, adjacent 5  $\mu$ m sections were stained with anti-VEGFR-3 alone. This staining confirmed that several of the PAL-E positive blood vessels are also positive for VEGFR-3.

D. VEGF-C, VEGFR-1, and VEGFR-2 in the intraductal carcinoma cells and its receptors in adjacent vessels

Polyclonal affinity-purified antibodies against human VEGF-C were used to stain the 8 intraductal carcinoma samples. All tested samples contained at least some VEGF-C, but considerable heterogeneity was observed in the intensity of staining and in the expression patterns. In some cases, most of the carcinoma cells were strongly positive for VEGF-C, while in others, only some carcinoma cells gave a staining signal. In contrast, very little or no staining was observed in the normal tissues surrounding the affected ducts, although weak signal was also obtained in unaffected normal ductal epithelium. Antigen blocking experiments indicated that the staining for VEGF-C was specific. The other VEGF-C receptor, VEGFR-2, as well as the other VEGF receptor (VEGFR-1), were both expressed in the same "necklace" vessels adjacent to the intraductal carcinoma cells.

E. VEGFR-3 positive vessels and VEGF-C in invasive breast carcinoma

Strongly-stained VEGFR-3 positive vessels were also present in all invasive ductal carcinomas and lobular carcinomas studied. The VEGFR-3 positive vessels did not appear to form any specific distribution pattern; most of these vessels were also immunoreactive for the PAL-E antigen. The intratumoral VEGFR-3 positive vessel density (median 21, range 9-56 vessels per hpf, n=12) was significantly elevated in the invasive breast carcinomas when compared with normal breast tissue (median 21 vs. 9;  $P < 0.0001$ , the Mann-Whitney test). Occasionally, invasion of the carcinoma cells into the VEGFR-3 positive lymphatic vessels could be observed.

Immunostaining for VEGF-C varied strongly among the invasive carcinomas studied (n=12). Some carcinoma cells were strongly positive for VEGF-C, while others stained very weakly or, in some cases, no staining was observed. Like in the intraductal carcinomas, very little or no staining was observed in the connective tissue in these sections.

The foregoing data reveals that VEGFR-3, which had otherwise appeared to be a predominantly lymphatic endothelial marker in most adult tissues, is very weakly expressed also in capillary endothelium of normal breast tissue. More

significantly, in intraductal carcinomas, a "necklace" pattern of strongly-stained VEGFR-3 positive vessels was detected lining the tumor-filled ducts. Most of these vessels expressed the blood vessel endothelial marker PAL-E and the basal lamina components laminin and collagen XVIII, but apparently had less pericytes/smooth muscle cells than blood vessels located further away from the tumor cells, as shown by staining using antibodies against SMA. These features suggest that the "necklace" vessels were undergoing angiogenesis. A second group of vessels lying a distance away from the affected ducts were positive for VEGFR-3 but very weakly positive for the basal lamina components and negative for PAL-E, suggesting that they are lymphatic vessels. These vessels also lacked SMA-positive pericytic components. Also in invasive breast carcinomas, VEGFR-3 was upregulated in PAL-E positive blood vessels, although the vessel patterns seen were more randomly organized in the connective tissue stroma around the tumor cells. The results indicate that VEGFR-3 expression is upregulated in breast carcinomas during angiogenesis associated with tumor growth. The highly elevated number of VEGFR-3 positive vessels found in carcinoma *in situ* is compatible with the hypothesis that the carcinoma cells produce factors, which activate the growth of blood vessels in the immediate vicinity of the carcinoma cells.

Since VEGF-C binds both VEGFR-3 and VEGFR-2 with high affinity, and since both intraductal and invasive carcinoma cells often stained positive for VEGF-C protein, this growth factor is a candidate growth factor for the VEGFR-3 and VEGFR-2 positive vessels in the carcinomas. These data are in agreement with another study, in which nearly half of thirty-five unselected malignant invasive tumors (including breast carcinomas, squamous cell carcinomas, lymphomas, melanomas, and sarcomas) contained VEGF-C mRNA in Northern blotting analyses. [See Salven *et al.*, *Am. J. Pathol.*, 153(1): 103-108 (July, 1998), incorporated herein by reference in its entirety.] Collectively, the data reported herein provide an indication for treatment of breast carcinomas and possibly other, non-lymphatic carcinomas with agents that block the VEGF-C mediated stimulation of VEGFR-3 and/or VEGFR-2. Contemplated blocking agents include: anti-VEGF-C antibodies; anti-VEGFR-3

antibodies; anti-VEGFR-2 antibodies; bispecific antibodies that bind to VEGFR-3 and either VEGFR-2 or VEGFR-1; soluble extracellular domain fragments of VEGFR-3 that will bind circulating VEGF-C; VEGF-C fragments and analogs that bind VEGFR-3 and/or VEGFR-2 and that inhibit activation of such receptors; VEGF-C

5 polypeptides, fragments, and analogs that bind VEGFR-3 and/or VEGFR-2 and that are conjugated to a suitable therapeutic agent; VEGFR-3 tyrosine kinase inhibitors; and small molecules that bind and inhibit these receptors. In addition, since VEGF-D binds both VEGFR-3 and VEGFR-2, it is contemplated that anti-VEGF-D antibodies and inhibitory VEGF-D fragments and analogs are suitable blocking agents. Human or  
10 humanized antibodies and fragments thereof are preferred, to the extent that antibody agents are selected for human therapy. Additionally, it is contemplated, as an additional aspect of the invention, to use any of the foregoing agents to evaluate mammalian tissue *in vitro* or *in vivo*, e.g., for the purposes of diagnosis and screening for malignancies and the spread of malignancies.

15 For any of the foregoing agents, it is contemplated that the agent may be further improved for diagnosis and screening by the attachment of a detectable label, including but not limited to radioisotopes (e.g.,  $^{14}\text{C}$ ,  $^{133}\text{I}$  and  $^{125}\text{I}$ ), chromophores (e.g., fluorescein, phycobiliprotien; tetraethyl rhodamine; enzymes which produce a fluorescent or colored product for detection by fluorescence; absorbance, visible color,  
20 or agglutination, which produces an electron-dense product for detection by electron microscopy); or electron dense molecules such as ferritin, peroxidase, or gold beads. Likewise, the agents may be further improved for therapeutic purposes by attachment (e.g., conjugation) or co-administration with molecules having anti-neoplastic properties, such as toxins of plant, animal, microbial, or fungal origin; radioisotopes;  
25 drugs; enzymes; and/or cytokines and other therapeutic proteins. (See, e.g., Pietersz & McKenzie, "Antibody Conjugates for the treatment of Cancer," *Immunological Reviews*, 129:57-80 (1992), incorporated by reference herein.

## EXAMPLE 29

### Anti-Flt4 antibodies for administration as a therapeutic to humans

#### A. Humanization of anti-Flt4 monoclonal antibodies

5 The biology of Flt4 as reported herein, e.g., in Example 28, indicates therapeutic uses for Flt4 inhibitors (antagonists) that block ligand-mediated signalling of the Flt4 receptor. Flt4-neutralizing antibodies comprise one class of therapeutics useful as Flt4 antagonists. Following are protocols to improve the utility of anti-Flt4 monoclonal antibodies as therapeutics in humans, by "humanizing" the monoclonal antibodies to improve their serum half-life and render them less immunogenic in human  
10 hosts (*i.e.*, to prevent human antibody response to non-human anti-Flt4 antibodies).

The principles of humanization have been described in the literature and are facilitated by the modular arrangement of antibody proteins. To minimize the possibility of binding complement, a humanized antibody of the IgG4 isotype is preferred.

15 For example, a level of humanization is achieved by generating chimeric antibodies comprising the variable domains of non-human antibody proteins of interest, such as the anti-Flt4 monoclonal antibodies described herein, with the constant domains of human antibody molecules. (See, e.g., Morrison and Oi, *Adv. Immunol.*, 44:65-92 (1989).) The variable domains of Flt4 neutralizing anti-Flt4 antibodies are  
20 cloned from the genomic DNA of a B-cell hybridoma or from cDNA generated from mRNA isolated from the hybridoma of interest. The V region gene fragments are linked to exons encoding human antibody constant domains, and the resultant construct is expressed in suitable mammalian host cells (*e.g.*, myeloma or CHO cells).

To achieve an even greater level of humanization, only those portions  
25 of the variable region gene fragments that encode antigen-binding complementarity determining regions ("CDR") of the non-human monoclonal antibody genes are cloned into human antibody sequences. [See, e.g., Jones *et al.*, *Nature*, 321:522-525 (1986); Riechmann *et al.*, *Nature*, 332:323-327 (1988); Verhoeyen *et al.*, *Science*, 239:1534-36 (1988); and Tempest *et al.*, *Bio/Technology*, 9:266-71 (1991).] If necessary, the  $\beta$ -  
30 sheet framework of the human antibody surrounding the CDR3 regions also is



modified to more closely mirror the three dimensional structure of the antigen-binding domain of the original monoclonal antibody. (See Kettleborough *et al.*, *Protein Engin.*, 4:773-783 (1991); and Foote *et al.*, *J. Mol. Biol.*, 224:487-499 (1992).)

5 In an alternative approach, the surface of a non-human monoclonal antibody of interest is humanized by altering selected surface residues of the non-human antibody, *e.g.*, by site-directed mutagenesis, while retaining all of the interior and contacting residues of the non-human antibody. See Padlan, *Molecular Immunol.*, 28(4/5):489-98 (1991).

10 The foregoing approaches are employed using Flt4-neutralizing anti-Flt4 monoclonal antibodies and the hybridomas that produce them, such as antibodies 9D9F9, to generate humanized Flt4-neutralizing antibodies useful as therapeutics to treat or palliate conditions wherein Flt4 expression is detrimental.

B. Human Flt4-Neutralizing Antibodies from phage display

15 Human Flt4-neutralizing antibodies are generated by phage display techniques such as those described in Aujame *et al.*, *Human Antibodies*, 8(4):155-168 (1997); Hoogenboom, *TIBTECH*, 15:62-70 (1997); and Rader *et al.*, *Curr. Opin. Biotechnol.*, 8:503-508 (1997), all of which are incorporated by reference. For example, antibody variable regions in the form of Fab fragments or linked single chain Fv fragments are fused to the amino terminus of filamentous phage minor coat protein pIII. Expression of the fusion protein and incorporation thereof into the mature phage coat results in phage particles that present an antibody on their surface and contain the genetic material encoding the antibody. A phage library comprising such constructs is expressed in bacteria, and the library is panned (screened) for Flt4-specific phage-antibodies using labelled or immobilized Flt4 as antigen-probe.

20

C. Human Flt4-neutralizing antibodies from transgenic mice

Human Flt4-neutralizing antibodies are generated in transgenic mice essentially as described in Bruggemann and Neuberger, *Immunol. Today*, 17(8):391-97 (1996) and Bruggemann and Taussig, *Curr. Opin. Biotechnol.*, 8:455-58 (1997).

- 5 Transgenic mice carrying human V-gene segments in germline configuration and that express these transgenes in their lymphoid tissue are immunized with an Flt4 composition using conventional immunization protocols. Hybridomas are generated using B cells from the immunized mice using conventional protocols and screened to identify hybridomas secreting anti-Flt4 human antibodies (e.g., as described above).

10 D. Bispecific antibodies

Bispecific antibodies that specifically bind to Flt4 and that specifically bind to other antigens relevant to pathology and/or treatment are produced, isolated, and tested using standard procedures that have been described in the literature. See, e.g., Pluckthun & Pack, *Immunotechnology*, 3:83-105 (1997); Carter *et al.*, *J.*

- 15 *Hematotherapy*, 4: 463-470 (1995); Renner & Pfreundschuh, *Immunological Reviews*, 1995, No. 145, pp. 179-209; Pfreundschuh U.S. Patent No. 5,643,759; Segal *et al.*, *J. Hematotherapy*, 4: 377-382 (1995); Segal *et al.*, *Immunobiology*, 185: 390-402 (1992); and Bolhuis *et al.*, *Cancer Immunol. Immunother.*, 34: 1-8 (1991), all of which are incorporated herein by reference in their entireties.

20

**EXAMPLE 30**

**Animal models to demonstrate the efficacy of anti-Flt4 therapies for treatment of cancers**

It is contemplated that any accepted animal for cancer therapies would be useful to demonstrate the efficacy of anti-Flt4 therapies for cancer treatment.

- 25 Exemplary models for demonstrating the efficacy for treatment of breast cancers, using standard dose-response studies, include those described in Tekmal and Durgam, *Cancer Lett.*, 118(1): 21-28 (1997); Moshakis *et al.*, *Br. J. Cancer*, 43: 575-580 (1981); and Williams *et al.*, *J. Nat. Cancer Inst.*, 66: 147-155 (1981). In addition to

murine models, dog and pig models are contemplated because at least certain anti-human Flt4 antibodies (*e.g.*, the 9D9 antibodies) also recognize Flt4 from dog and pig. Tumor size and side effects are monitored to demonstrate therapeutic efficacy versus controls.

5                   While the invention here has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice  
10                   within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth and as follows in the scope of the appended claims.